THE BIOCHEMICAL JOURNAL

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Purification of the Nicotinic Acid Hydroxylase System of *Pseudomonas fluorescens* KB1

By A. L. HUNT*
Department of Biochemistry and Medical Research Council Unit for Research in Cell Metabolism, University of Oxford

(*Received* 28 *August* 1958)

The nicotinic acid hydroxylase system of *Pseudomonas fluorescens* KB1 has previously been shown to be an adaptive enzyme which hydroxylates nicotinic acid in the C-6 position with the uptake of one molecule of oxygen for two molecules of nicotinic acid (Hughes, 1955). The reaction initiates the breakdown of nicotinic acid to ammonia and formic, acetic, lactic and malic acid according to the reactions described by Behrman & Stanier (1957). In cell-free extracts nicotinic acid is converted quantitatively into 6-hydroxynicotinic acid and this compound is not further metabolized. The hydroxyl group added is derived from water and not molecular oxygen, indicating that the reaction probably involves the addition of water followed by dehydrogenation with molecular oxygen as hydrogen acceptor (Hunt, Hughes & Lowenstein, 1957, 1958). The enzyme is attached to insoluble particulate components of the cell. This paper is concerned with the purification of the enzyme, together with the components of an associated electron-transport chain, by deoxycholate extraction of the particles, followed by ammonium sulphate fractionation. Preliminary findings of this work have been communicated (Hunt, 1958).

METHODS

Growth of organisms. *P. fluorescens* KB1 was grown with aeration at 25° in an inorganic salt medium containing KH_2PO_4, 6·8 g., NH_4Cl, 1·0 g., mineral supplement (Behrman & Stanier, 1957), 1·0 ml., $MgSO_4,7H_2O$, 0·5 g., yeast extract (Oxo Ltd.), 0·2 g. and nicotinic acid, 2·5 g. per litre of demineralized water. The solution was adjusted to pH 6·8 with solid NaOH. Subcultures were maintained on slopes containing in addition 2% of agar. For the preparation of large quantities of cells 40 l. batches of medium were inoculated with 4 l. of a logarithmic-phase culture, and growth was allowed to continue under forced aeration in the stainless-steel container of a commercial washing machine (Hoover Ltd.) fitted with a baffle plate and a stainless-steel cover. The airflow was maintained at levels suboptimum for maximum rates of growth. Small quantities of tributyl citrate were added to minimize foaming. Under the conditions used, cell densities reached 3–4 g. wet wt./l. after growth for 10–12 hr. The cells were harvested in a Sharples centrifuge before the onset of the stationary phase. A further 20 g. of nicotinic acid was added before harvesting, and the culture was agitated with a stream of air during the harvesting period. The washed cells were stored at −15° before disintegrating. Cells with optimum activity assumed a deep red colour when frozen at −15°.

Preparation of cell-free extracts. The frozen cell masses were crushed at −20° without the use of abrasive in a Hughes press (Hughes, 1951), modified to accommodate 70 g. of cells; the cylinder measured 4 cm. in diameter. The press was operated by dropping a steel weight of 50 kg. a distance of 1·8 m. down a cylinder mounted above the piston. The crushed cells were homogenized in 2 vol. of ice-cold 0·066 M-sodium potassium phosphate buffer, pH 7·20 (Clarke, 1928), in a glass homogenizer (Potter & Elvehjem, 1936). Approx. 1 mg. of crystalline deoxyribonuclease was added to 500 ml. of the buffer to reduce the viscosity of the homogenate. The mixture was allowed to stand at 0–2° before centrifuging.

Enzyme assays. Oxygen uptake was measured in a conventional Warburg apparatus at 30°. Cups contained 1·0 ml. of 0·066 M-sodium potassium phosphate buffer, pH 7·20, 0·3 ml. of 0·2 M-substrate, enzyme and water to a final volume of 2·0 ml.

Nicotinic acid hydroxylase activity was assayed spectrophotometrically by the reduction of ferricyanide. Cuvettes contained 1·0 ml. of 0·066 M-sodium potassium phosphate buffer, pH 7·20, 0·3 ml. of 0·2 M-potassium nicotinate, 0·3 ml. of 0·1 M-potassium ferricyanide, enzyme and water to a final volume of 3·0 ml. The blank cell contained water instead of substrate. The reaction was started by the addition of enzyme and reduction was followed at 450 mμ in a Carey recording spectrophotometer. The unit of activity is described as the reduction of 1 μmole of ferricyanide in 10 min. at 25°.

Cytochrome oxidase, reduced pyridine nucleotide cytochrome *c* reductase, reduced pyridine nucleotide dehydrogenase and succinoxidase activities were assayed according to procedures described by Mackler & Green (1956).

Spectrophotometric measurements. Cytochrome spectra were obtained in a Carey wavelength-recording spectrophotometer, model 14M-50, with standard 10 mm. quartz cells or 40 mm. Perspex cells fitted with quartz windows. Difference spectra (reduced minus oxidized) were recorded with the reference cell containing enzyme fully oxidized by the addition of a trace of potassium ferricyanide, and the sample cell containing enzyme reduced by the addition of substrate or a crystal of sodium dithionite. Pyridine and cyanide haemochromogens were prepared as follows. The

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enzyme solution was mixed with an equal volume of 0·4 N-NaOH and then an equal volume of freshly distilled pyridine or a small quantity of potassium cyanide. The slight precipitate that formed was removed by centrifuging. Difference spectra were recorded as described above. Total haem-protein concentrations were obtained from the above difference spectra with the μmolar extinction coefficients recommended by Chance (1952). Flavin assays were based on the difference spectrum of the enzyme at 460 mμ according to the method of Green, Mii & Kohout (1955).

Chemical estimations. Total N was determined by a micro-Kjeldahl method; the value for total N was multiplied by 6·25 to give an approximate value for protein N. Total iron was estimated with *o*-phenanthroline (Sandell, 1944) after digestion with HNO_3. Samples were well dialysed against water before digestion and contained only trace amounts of phosphorus.

Sedimentation. This was observed in a Svedberg oil-turbine ultracentrifuge, equipped with Baldwin's (1953) modification of the diagonal-schlieren optical system of Philpot (1938). Measurements were made at a rotor speed of 900 rev./sec., and the rotor temperature was usually 26–28°. Sedimentation coefficients were computed by the method of Cecil & Ogston (1948).

Zone electrophoresis. This was performed in a starch column according to the method of Smithies (1955). The purified enzyme was introduced into a 2–3 mm. slit cut in the centre of the block, in the form of a slurry with powdered starch. After electrophoresis the two halves of the block were removed from the carrier, and each was cut longitudinally and then in halves with a tissue slicer. Corresponding portions of the block were each stained to detect enzyme activity, haem components, flavin pigments and protein, as follows. For hydroxylase activity the starch strip was painted with a solution of the standard assay solution, containing nicotinate, ferricyanide and phosphate buffer. The strip was incubated at room temperature for 15 min. and then painted with a dilute solution of $FeCl_3$ in water. Areas of ferricyanide reduced by nicotinate hydroxylase activity appeared as deposits of ferric ferrocyanide. Flavin pigments were detected by examining the strip under ultraviolet irradiation. Haem pigments were detected by painting the strip with a solution of benzidine in acetic acid, and then with hydrogen peroxide, according to Smithies (1955). Protein was detected by staining with a solution of Amido-Black in methanol–acetic acid, also according to the procedure described by Smithies (1955).

RESULTS

Distribution of activity in whole homogenates

Whole homogenates of crushed cells prepared as indicated above were centrifuged at 60 000 *g* for 45 min. in rotor 30 of the Spinco preparative ultracentrifuge. Under these conditions the insoluble cellular material separated into well-defined particulate layers below a clear-yellow supernatant. The particulate residue contained two main constituents, a heavier brown–pink layer and an upper bright-pink layer. After removal of the supernatant, the latter layer could be removed easily into fresh buffer with the aid of a curved spatula. This material was rehomogenized, recentrifuged at 25 000 *g* for 45 min., and resuspended in half the original volume of fresh buffer. The nature of these fractions and the distribution of nicotinic acid hydroxylase activity in the whole extracts will be described fully elsewhere. The resuspended pink upper layer resembled the cell-wall and protoplast-membrane fraction of the cell (cell wall–membrane fraction), and contained the bulk of the hydroxylase activity of the whole extract. Removal of the soluble inactive proteins of the original homogenate resulted in a three- to five-fold purification of nicotinic acid hydroxylase activity in the cell wall–membrane fraction, based both on oxygen uptake and ferricyanide reduction. This fraction also oxidized succinate, malate, lactate and reduced pyridine nucleotides, but 6-hydroxynicotinic acid was not metabolized. The opaque suspensions could be clarified by suspension in a 30 % (w/v) solution of serum albumin (The Armour Laboratories), which allowed spectrophotometric observations to be made. Such suspensions retained the full enzymic activity of the original material. Under these conditions, marked reduction of cytochrome and flavin components could be observed on the addition of nicotinate, succinate or reduced pyridine nucleotides. Thus the cell wall–membrane fraction contains electron-transport chains for the oxidation of these substances, as do mitochondrial systems in higher organisms.

Oxygen could be replaced by ferricyanide, methylene blue and indophenol dyes as electron acceptors in the hydroxylation of nicotinic acid.

Elution of nicotinic acid hydroxylase activity

The cell wall–membrane fraction may be washed repeatedly by centrifuging and resuspension in buffer without loss of activity. However, treatment with buffer containing a detergent released varying amounts of activity into the supernatant. Of reagents tested, sodium deoxycholate proved most effective and, under optimum conditions, released up to 80 % of the bound activity. The effect of detergent treatment is shown in Table 1. Deoxycholate also released considerable quantities of flavin and haem pigments. Both the yield of hydroxylase activity and the latter pigments varied greatly with the ratio of protein to deoxycholate concentration. These relationships are illustrated in Table 2. Increasing deoxycholate released increasing amounts of hydroxylase, flavin and haem pigments up to a ratio of approx. 1 mg. of deoxycholate to 12 mg. of protein. Beyond this level increasing deoxycholate released increasing amounts of flavin pigments but little further hydroxylase or haem pigment.

Nicotinic acid hydroxylase activity in these eluates coupled rapidly with ferricyanide, indophenols and methylene blue as hydrogen acceptors

Table 1. *Effect of detergents on the cell wall–membrane fraction*

Suspensions of the cell wall–membrane fraction (4·0 ml., 162 mg. of N × 6·25) were homogenized in 0·066 M-sodium potassium phosphate buffer, pH 7·20, with detergents as indicated [sorbitan mono-oleate (Tween; Honeywill and Stein Ltd., London); polyethylene glycol complex (Gemex; British Drug Houses Ltd.)]. The homogenates were centrifuged at 60 000 *g* for 45 min. The residues were resuspended in the same volume of fresh buffer. Activities represent total units based on ferricyanide reduction.

	Phosphate	0·3% (w/v) Tween	0·3% (w/v) Gemex	0·3% (w/v) Deoxycholate
Residue	7300	7200	7200	2000
Supernatant	0	400	250	8000

Table 2. *Effect of deoxycholate concentration on the elution of nicotinic acid hydroxylase activity, flavin and haem pigments from the cell wall–membrane fraction*

Suspensions of the cell wall–membrane fraction (8·0 ml., 300 mg. of N × 6·25) were homogenized in 0·066 M-sodium potassium phosphate buffer, pH 7·20, containing varying amounts of sodium deoxycholate as indicated. The homogenates were centrifuged at 60 000 *g* for 45 min. at 0°. Hydroxylase activity, total protein, flavin and haem pigments were assayed in the supernatant. Enzyme activity is expressed as total units of the fraction based on ferricyanide reduction. Figures for haem-protein and flavin are given as μm-moles.

	Concn. of deoxycholate (mg./ml.)			
	0	1·5	3·0	6·0
Cell wall–membrane fraction	4000	—	—	—
Units eluted	0	2000	3200	3600
Protein eluted (mg.)	0	40	61	76
Specific activity of the eluate	—	50	52	47
Total haem eluted	0	5·1	9·6	9·2
Total flavin eluted	0	56·0	80·0	110·0

but not with oxygen. Deoxycholate inhibited oxygen uptake completely in other fractions and could be removed by prolonged dialysis from the above eluates, but this treatment usually resulted in complete loss of hydroxylase activity. The enzyme was similarly unstable in the presence of deoxycholate; activity was lost completely overnight at −15°.

Electron micrographs of the cell wall–membrane fraction before and after extraction are shown in Figs. 1 and 2. Although retaining the shape of the original structure, the treated material was less electron-dense and lay very flat on the grid, indicating that deoxycholate did not disintegrate the fraction but eluted protein and other constituents from further structural material.

Hydroxylase activity may also be released into a supernatant fraction by ultrasonic disruption, but

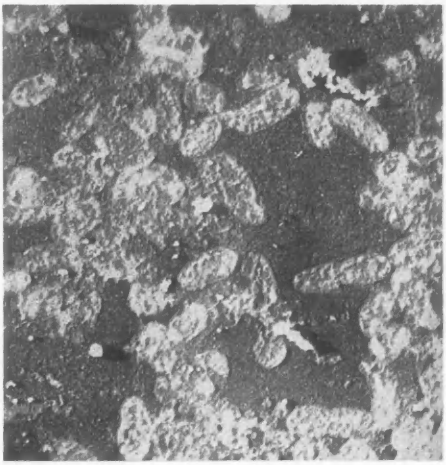

Fig. 1. Electron micrograph of the cell wall–membrane fraction. Shadowed with platinum.

Fig. 2. Electron micrograph of the cell wall–membrane fraction treated with deoxycholate. Shadowed with platinum.

such activity remains attached to polydisperse fragments of the larger particle fraction, and may be sedimented at ultracentrifugal speeds.

Fractionation of deoxycholate extracts

In the analytical ultracentrifuge, deoxycholate extracts showed slow-moving polydisperse components. Whereas hydroxylase activity remained in the supernatant after centrifuging at 60 000 *g* for 60 min., the behaviour of the enzyme in the presence of low concentrations of ammonium sulphate indicated that activity was probably associated with finely divided particulate material. This latter property formed the basis of further purification of the enzyme from deoxycholate extracts. The following procedure was used (Fig. 3).

The washed cell wall–membrane fraction was resuspended in ice-cold 0·066 M-sodium potassium phosphate buffer, pH 7·20 (40–50 mg. of N × 6·25/ml.). The suspension was homogenized in a glass Potter–Elvehjem homogenizer, with the addition of 1·2% (w/v) deoxycholate solution, previously adjusted to pH 7·20, to give a final concentration of 1·0 mg. of deoxycholate/12·5 mg. of N × 6·25. The homogenate was centrifuged at 60 000 *g* for 45 min. at 0° and the orange–yellow supernatant transferred to a flask immersed in ice. The residue was usually re-extracted in a similar manner and the supernatants were pooled. Nicotinic acid hydroxylase activity was assayed in the supernatant fraction immediately after preparation. Saturated ammonium sulphate solution containing mM-disodium ethylenediaminetetra-acetate, and adjusted to pH 7·20 by glass electrode with aq. NH_3 soln. (sp.gr. 0·880), was then added dropwise with stirring until the concentration reached 15% saturation. The mixture was allowed to stand for 30 min. at 0°. A bulky white precipitate formed at this stage and was removed by centrifuging at 0°. The latter contained traces of hydroxylase activity and was discarded. Further ammonium sulphate was added to the supernatant to 35% saturation, and the precipitate recovered by centrifuging at 0°. The deep-yellow supernatant solution was discarded. The precipitate was pink in colour and contained the bulk of the hydroxylase activity of the deoxycholate extract. This material redissolved in fresh buffer to give an optically clear brick-red solution. Hydroxylase activity was assayed in a diluted portion of the solution and the

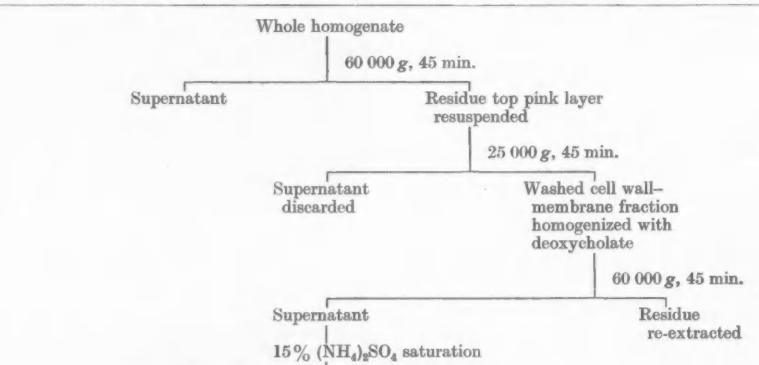

Fig. 3. Purification scheme.

Table 3. *Purification of the nicotinic acid hydroxylase system*

Fraction	10^{-3} × Total units	Protein N × 6·25 (mg.)	Specific activity (units/mg. of N × 6·25)
Whole homogenate	400	32 000	12·5
High-speed supernatant	50	17 500	2·8
Cell wall–membrane fraction	261·5	7 500	35·0
Deoxycholate extract	130	2 000	65·0
15–35% $(NH_4)_2SO_4$ fraction	90	150	600

remainder dialysed at 0–2° against 1000 vol. of the same buffer for 10–12 hr. Approx. 10% of the activity was lost on dialysis. Most preparations averaged 500–600 units of activity/mg. of protein, based on ferricyanide reduction, representing a 50-fold purification over the original whole extract. The activities at various stages of purification are indicated in Table 3.

Properties of the purified enzyme

The enzyme hydroxylated nicotinic acid rapidly with ferricyanide, indophenol dyes and methylene blue as electron acceptors. In well-dialysed preparations oxygen could also act as the terminal electron acceptor although oxygen uptake was inhibited greatly by trace amounts of deoxycholate. Purification based on oxygen uptake averaged 15-fold. Oxygen uptake was stimulated markedly by the addition of phenazine methosulphate, and was inhibited completely by mM-cyanide and 50% by mM-sodium azide. Cyanide was not inhibitory in the early stages of reduction with ferricyanide as electron acceptor, but it inhibited markedly as the reaction progressed. Inhibition in this case was not enhanced by preincubation with cyanide or with cyanide plus 6-hydroxynicotinic acid.

Nicotinate was the only substrate attacked by the preparation (Table 4). Although the cell wall–membrane fraction oxidized succinate, malate, lactate and reduced pyridine nucleotides, these substrates were not metabolized in the purified preparation. Addition of reduced pyridine nucleotides also failed to reduce the flavin and haem pigments in the fraction. Reduced mammalian cytochrome *c* was not reoxidized, nor was the oxidized compound reduced in the presence of nicotinate. Although a purified cytochrome *c* preparation has been obtained from this organism (Kogut, 1957), no attempt has been made to assay cytochrome reductase or cytochrome oxidase with this compound as donor or acceptor. Oxidative phosphorylation with nicotinate as substrate could not be demonstrated.

Addition of nicotinate in the absence of electron acceptors caused a marked change in the spectrum of the solution. This change was enhanced by the addition of a trace of potassium cyanide and still further by addition of sodium dithionite. The difference spectrum (reduced minus oxidized) showed absorption maxima at 560, 553, 550, 530, 523 and 428 mμ, with a shoulder at 510–515 mμ

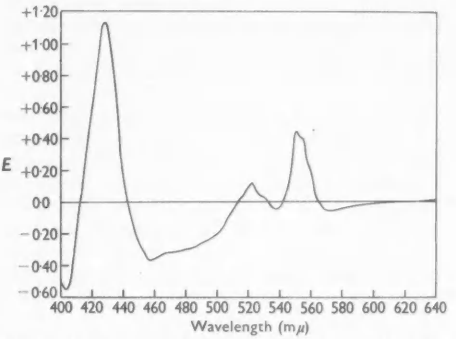

Fig. 4. Difference spectrum (reduced minus oxidized) of the purified nicotinic acid hydroxylase complex, reduced by the addition of sodium dithionite. Cuvettes (40 mm.) contained 3·3 mg. of protein/ml.

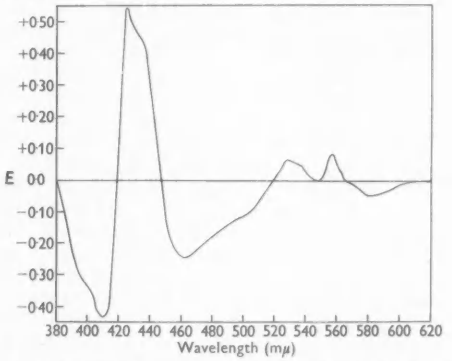

Fig. 5. Difference spectrum (reduced minus oxidized) of the cyanide haemochromogen derivative of the purified nicotinic acid hydroxylase complex.

Table 4. *Reaction of the cell wall–membrane fraction and the purified enzyme with oxygen and phenazine methosulphate*

Warburg cups contained 1·0 ml. of 0·066M-sodium potassium phosphate buffer, pH 7·20, 0·3 ml. of 0·2M-substrate, water and 50 mg. of N × 6·25 of cell wall–membrane fraction, 4·2 mg. of N × 6·25 of the purified enzyme and 2·9 mg. of phenazine methosulphate where indicated. Final volume 2·0 ml. Temperature 30°; atmosphere air. Figures represent μmoles of O_2 uptake/hr./mg. of N × 6·25, corrected for blank respiration.

	Nicotinate	Succinate	Malate	Lactate	Nicotinate + phenazine methosulphate	Succinate + phenazine methosulphate
Cell wall–membrane fraction	2·6	1·1	1·3	0·1	—	—
Purified enzyme	34	0	0	0	46	0

and minima at 574, 537 and 460 mμ. A tracing of the difference spectrum recorded on the Carey spectrophotometer is presented in Fig. 4. The reduced complex was rapidly reoxidized by the addition of 0·1 mM-hydrogen peroxide. The difference spectrum of the reduced cyanide haemochromogen derivative of the enzyme preparation exhibited maxima at 556, 537, 526 and 427 mμ, with minima at 547 and 460–467 mμ (Fig. 5). The corresponding pyridine haemochromogen exhibited maxima at 552 mμ, a broad peak between 517 and 522 mμ, and at 415 mμ. After treatment in the dark with pure carbon monoxide, the dithionite-reduced enzyme exhibited maxima not significantly different from those obtained without carbon monoxide treatment. The enzyme contained 1·2 μm-moles of haem and 1·8–2·8 μm-moles of flavin/mg. of N × 6·25, in several preparations. Only half of the flavin was reduced in the presence of nicotinate. The preparation also contained 7 moles of non-haem iron/mole of haem iron, based on *o*-phenanthroline colour.

In the analytical ultracentrifuge, the purified enzyme showed rapidly moving polydisperse components together with slower heterogeneous peaks sedimenting at S_{20} 9·6, 7·3 and 2·0.

The preparation was also subjected to zone electrophoresis in a starch block according to the method of Smithies (1955). Detection of haem, flavin, hydroxylase activity and protein was carried out as indicated under Methods. At pH 8·68 in 0·03 M-sodium potassium phosphate buffer, the enzyme activity, haem components and some of the flavin pigment failed to migrate more than a few millimetres in the starch gel. Three other protein components, one of which was a second flavoprotein, moved in the direction of the cathode. Varying the buffer concentration and the pH of the solution failed to produce any variation in the pattern except in the degree of resolution of the mobile components. It must be assumed therefore that either the enzyme, haem and flavin complex are uncharged at the pH values used, or that the starch granules prevent the movement of the complex in the electrical field.

DISCUSSION

Together with the demonstration that the hydroxyl group added to the C-6 position is derived from water, the above evidence affords evidence for the mechanism of nicotinic acid hydroxylation. This reaction sequence most probably involves addition of water to the ring structure followed by dehydrogenation with oxygen as hydrogen acceptor and takes place through a flavin enzyme and the electron-transport system of the cell. The intermediate steps in the reaction are unknown. Harary (1957), using the chemical analogy of the oxidation of *N*-methylpyridinium hydroxide to *N*-methylpyridone, suggested the reaction involved the addition of the hydroxyl group to the C-2 position, with the formation of the 2-hydroxyl-1:2-dihydro compound, followed by dehydrogenation to the pyridone and rearrangement. It is of interest that the present preparation failed to metabolize the 2-fluoro-, the *N*-methyl- and the 5-fluoro-derivatives of nicotinic acid. Similarly nicotinic acid *N*-oxide was not converted into the 6-hydroxy compound, nor reduced to nicotinic acid in the presence of reduced benzyl-Viologen, suggesting that this compound is not involved in the reaction.

Deoxycholate has been used extensively in the elution of particulate enzymes and in the comminution of larger cell fragments of animal-cell preparations (Green, 1956–57). Many components of the mitochondrial system have been separated with varying concentrations of deoxycholate and salt. In particular, reduced pyridine nucleotide and succinic acid oxidase complexes have been separated and evidence for separate electron-transport chains for these sources described (Rabinowitz & De Bernard, 1957). However, there have been few reports of the use of this reagent for similar purposes in micro-organisms. King & Cheldelin (1957) recently reported the elution of glucose oxidase from subcellular particles of *Acetobacter suboxydans*.

The cytochrome system and oxidative phosphorylation enzymes appear to be localized in the protoplast membrane or an equivalent structure in the bacterial cell. This structure approximates the mitochondrion of animal cells. The present report adds supporting evidence to the concept that organization of the electron-transport system in the bacterial membrane may be a counterpart of that found in the animal mitochondrion. The hydroxylase system, isolated from the structure under the conditions of deoxycholate and salt concentration described, is associated with an electron-transport chain devoid of succinoxidase and reduced pyridine nucleotide oxidase activity. The nature of the purified complex is under further investigation, but preliminary electrophoresis experiments indicate the presence of a complex of haem, flavin and hydroxylase activity, and several other components, one of which is a second flavoprotein.

There have been several reports of the cytochrome components of *Pseudomonas* species. Smith (1955) described absorption maxima at 424, 523, 560 and 580 mμ for whole cells of *P. fluorescens*. Vernon (1956*a*, *b*) described the purification and properties of cytochromes from *Pseudomonas denitrificans* and a further unidentified species. These components were identified as cytochrome *c* with maxima at 550, 520 and 416 mμ, cytochrome c_1 with maxima at 553, 523 and 419 mμ and cytochrome b_1 with maxima at 559, 528 and 426 mμ.

Kogut (1957) also described the purification of the '553 cytochrome' from *P. fluorescens* KB1. The difference spectrum (reduced minus oxidized) of the present complex also indicates the presence of cytochromes *c*, c_1 and b_1. The combined Soret band, however, is displaced several mμ towards longer wavelengths than would be expected from the combined bands of these components alone. The cyanide and pyridine haemochromogens confirm the identity of these components although the additional absorption at 435 mμ in the cyanide compound does not correspond to these compounds.

No evidence for a typical cytochrome oxidase component could be obtained in this preparation. Chance & Smith (1955) reported the existence of an unusual oxidase component in *Staphylococcus albus*. The reduced compound had absorption maxima at 553 and 424–430 mμ, and reacted with carbon monoxide to give a derivative with maxima at 567, 535 and 415 mμ. Recent studies of Bartsch & Kamen (1958) with the purified compound indicate in addition minima at 485 and 640 mμ, and a Soret maximum at 432 mμ. Although the present reduced complex has maxima at 553 and at 428 mμ, no carbon monoxide derivative could be formed under the conditions used by these workers.

Cytochrome *c* peroxidase has been suggested as a terminal respiratory enzyme in cultures of *P. fluorescens* grown under anaerobic conditions in the presence of nitrate (Lenhoff & Kaplan, 1956). This activity has not been fully investigated in the present complex, but the reduced enzyme is rapidly reoxidized by low concentrations of hydrogen peroxide, suggesting the presence of peroxidase activity. Cytochrome b_1 of *Pseudomonas* is itself autoxidizable but cyanide-insensitive, and the reduced cytochrome c_1 is only very slowly oxidized in air. Thus in the absence of further evidence it must be assumed that terminal respiration in the purified complex is mediated by a peroxidase enzyme of the type described by Lenhoff & Kaplan (1956).

SUMMARY

1. The nicotinic acid hydroxylase system of *Pseudomonas fluorescens* KB1 is associated with a particulate fraction, resembling the cell wall and protoplast membrane of the organism, in homogenates of crushed cells.

2. The enzyme has been eluted by deoxycholate extraction of the particulate fraction and further purified by ammonium sulphate fractionation. Overall purification averaged 50-fold, based on ferricyanide reduction.

3. The purified enzyme hydroxylated nicotinic acid with oxygen, ferricyanide, methylene blue, indophenol dyes and phenazine methosulphate as electron acceptors.

4. Succinate and reduced pyridine nucleotides were not oxidized by the preparation, although these compounds were readily utilized by the parent particulate fraction.

5. The purified preparation contained cytochromes *c*, c_1 and b_1 and flavoprotein components, which were rapidly reduced on the addition of nicotinic acid. Terminal oxidation is tentatively attributed to cytochrome *c* peroxidase activity.

6. The mechanism of nicotinic acid hydroxylation and the organization of the bacterial respiratory chain are discussed.

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Studies on the Adenosine Triphosphate–Phosphate Exchange and the Hydrolysis of Adenosine Triphosphate Catalysed by a Particulate Fraction from the Mosquito

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The citric acid cycle is known to be connected with the esterification of inorganic phosphate both in mammalian (Lehninger, 1954) and in insect (Sacktor, 1954; Lewis & Slater, 1954; Gonda, Traub & Avi-Dor, 1957) respiratory particles. In addition to the net synthesis of adenosine triphosphate, liver mitochondria and submitochondrial fractions also catalyse an exchange between adenosine triphosphate and labelled inorganic phosphate (^{32}P) in the absence of apparent respiration (Boyer, Luchsinger & Falcone, 1956; Swanson, 1956; Cooper & Lehninger, 1957). Strong evidence exists for a functional association of this exchange reaction with oxidative phosphorylation and 2:4-dinitrophenol-stimulated adenosine triphosphatase activity.

In a continuation of a study on the oxidative and phosphorylative metabolism of insects and on the mode of action of insecticides, the properties of the enzyme systems catalysing the adenosine triphosphate–^{32}P exchange and the hydrolysis of adenosine triphosphate have been investigated in preparations obtained from the mosquito *Aedes aegypti* L. The effect of various inhibitors and of 1:1:1-trichloro-2:2-di-(*p*-chlorophenyl)ethane on these enzymic activities has been tested.

EXPERIMENTAL

Insect colonies. *Aedes aegypti* L. were reared as described before (Gonda *et al.* 1957). Houseflies (*Musca domestica*) were cultivated by the Peet–Grady method as modified by Tahori (1955).

Materials. All nucleotides were products of Sigma Chemical Co. Fluoropyruvic acid was synthesized according to Blank, Mager & Bergmann (1955). Crystalline 1:1:1-trichloro-2:2-di-(*p*-chlorophenyl)ethane (DDT), m.p. 108°, was used.

Human serum albumin of 85% purity (containing 15% of globulin) prepared according to Nitschmann, Kistler & Lergier (1954) was obtained from Marcus Memorial Institute for Plasma Drying and Fractionation, Tel-Aviv. Solutions of the albumin were freshly prepared before use and adjusted by 0·5 M-2-amino-2-hydroxymethylpropane-1:3-diol (tris) buffer to pH 7·4. Tris and phosphate buffers used were tris–HCl and NaH_2PO_4–Na_2HPO_4 mixtures respectively. They were prepared according to Colowick & Kaplan (1955).

$Na_2H^{32}PO_4$ was obtained from The Radiochemical Centre, Amersham, Bucks. The neutralized stock solution contained approx. 1·5 μc/ml. and gave $1{\cdot}5 \times 10^5$ counts/min./ml. in a thin mica-window Geiger counter tube. The radioactivity was measured by evaporating a portion (0·2 ml.) of the ten-times diluted stock solution to dryness on a planchet of 2 cm.^{2} surface under an infrared lamp.

All other chemicals were of analytical grade.

Isolation of respiratory particles. Respiratory particles from the mosquito were obtained as described by Gonda *et al.* (1957), except that albumin was omitted from the isolation medium. The standard isolation medium (5 ml./g. of mosquito) contained: sucrose 0·33 M and ethylenediaminetetra-acetic acid (EDTA), 0·1 mM, adjusted to pH 7·4 with NaOH. The particles were washed twice with the isolation medium (70 ml. each time), and resuspended in 0·33 M-sucrose. Respiratory particles were also prepared from whole houseflies by the same method. Guinea-pig liver mitochondria were prepared according to the method of Schneider (1948). Unless otherwise indicated, the respiratory particles obtained from the mosquito were used in the following experiments.

Methods. The exchange between ATP and ^{32}P was assayed by a method based on the procedure described by Swanson (1956). The composition of the reaction mixture (total volume 1·5 ml.) is described in Table 1. Incubation was carried out in Pyrex test tubes in a water bath at 28°. The reaction was terminated by the addition of 4·5 ml. of 5% (w/v) trichloroacetic acid and the acidified sample centrifuged to remove protein. A portion (1 ml.) of the supernatant was used for the determination of the phosphate liberated from ATP (ATPase activity). To a 3 ml. portion of the supernatant 1 ml. of an aqueous suspension of acid-washed Norit A (equivalent to approx. 25 mg. dry wt.) was added. The charcoal was separated by centrifuging, washed once with 10 ml. of trichloroacetic acid (5%, w/v), twice with 10 ml. of water and resuspended in a small volume of ethanol. The ethanol suspension of the charcoal was transferred quantitatively to planchets of 2 cm.^{2} surface and dried under an infrared lamp. The radioactivity of the nucleotides adsorbed on the charcoal was counted in a thin mica-window Geiger counter tube and was expressed as counts/min./total nucleotide fraction. The counts measured in a control, in which trichloroacetic acid was added before the particle preparation, were deducted from the experimental values (as a rule the counts in the control exceeded only slightly the background counts).

A correction for the absorption of the charcoal layer was obtained from an empirical calibration curve which was prepared by evaporating samples of ^{32}P in the presence and in the absence of 25 mg. of Norit A. The specific activity of

Table 1. *Effect of the composition of the isolation medium on adenosine triphosphate–^{32}P exchange and adenosine triphosphatase activity*

Composition of isolation medium was as indicated. The isolated particles were washed twice with 0·33 M-sucrose. Composition of the 'standard' assay medium: tris buffer (10 mM), pH 7·4, phosphate buffer (2·7 mM), pH 7·4, 0·2 ml. of the stock solution of ^{32}P, sucrose (0·25 M), ATP (7 mM), human serum albumin 1 % (w/v), EDTA (mM), respiratory particles from mosquito (equivalent approx. to 0·2 mg. of N/ml. of assay medium). Total volume: 1·5 ml. Enzymic activities were measured as described under Methods. Time of incubation: 10 min. Temp. 28°. ΔP: inorganic phosphate liberated (μmoles).

Composition of isolation medium	Concn. (M)	ΔP		Total counts in nucleotides/min.	
		Albumin and EDTA omitted	'Standard' assay medium	Albumin and EDTA omitted	'Standard' assay medium
Sucrose	0·33	2·8	2·0	20	60
Sucrose EDTA	0·33 0·0001	2·2	1·8	30	3034

Table 2. *Effect of the composition of the washing medium*

Composition of isolation medium: sucrose 0·33 M, EDTA 0·1 mM. Composition of washing medium was as indicated in the Table. Other conditions of the experiment were as described in Table 1.

Composition of washing medium	Concn. (M)	ΔP		Total counts in nucleotides/min.	
		Albumin and EDTA omitted	'Standard' assay medium	Albumin and EDTA omitted	'Standard' assay medium
Sucrose	0·33	2·1	1·8	29	3100
Sucrose Albumin	0·33 1 % (w/v)	1·9	1·4	70	2530
Sucrose EDTA	0·33 0·001	2·3	1·8	36	3150
Sucrose EDTA Albumin	0·33 0·001 1 % (w/v)	1·2	1·4	2980	3220

Table 3. *Effect of the composition of the assay medium*

Respiratory particles were isolated and washed as described under Methods. Standard assay medium, but albumin and EDTA were omitted if not indicated. Other conditions were as described in Table 1.

Additions			
Albumin (%, w/v)	EDTA (mM)	ΔP	Total counts in nucleotides/min.
None	None	1·8	18
	0·1	1·6	20
	1·0	1·0	18
	10·0	0·4	16
0·5	None	1·8	27
1·0		1·7	26
2·0		1·7	35
1·0	0·1	1·6	208
1·0	1·0	1·0	2870
1·0	10·0	1·0	3400

^{32}P decreased during the time of incubation owing to the liberation of unlabelled phosphate from ATP. The magnitude of the change in the specific activity of ^{32}P varied under different conditions according to the ATPase activity. Therefore the experimentally found radioactivity of the nucleotide fraction was multiplied by the factor: average concentration of inorganic phosphate during the time of reaction/concentration of inorganic phosphate at zero time.

Nitrogen was determined according to Johnson (1941), and inorganic phosphate by the method of Fiske & Subbarow (1925).

RESULTS

Effect of the composition of the isolation, washing and assay media

Previous workers have shown that the addition of serum albumin and EDTA is necessary for the oxidative phosphorylation in insect-particle preparations (Sacktor, 1954; Lewis & Slater, 1954; Gonda *et al.* 1957). Therefore the effect of these substances on the two phosphorylative processes was studied. The composition of the medium was varied in: (*a*) isolation of the particles from the tissue; (*b*) washing of the particles; (*c*) assay of ATP–^{32}P exchange. The data presented in Tables 1–3 show that the presence of EDTA (0·1 mM) in the medium used for the isolation of the mosquito respiratory particles from the tissue was

required for a significant incorporation of ^{32}P into ATP under otherwise optimum conditions. Furthermore, either the assay medium or the sucrose solution used for the washing of the particles had to be supplemented with both EDTA and albumin [optimum concentrations: mM for EDTA and at least 1% (w/v) for albumin]. Neither of the two agents singly facilitated the ATP–^{32}P-exchange reaction. With albumin and EDTA in the assay medium the addition of these substances to the washing medium was unnecessary and the addition of albumin was even slightly inhibitory. Further, EDTA at high concentrations inhibits the hydrolysis of ATP, whereas albumin has no effect on ATPase activity. The requirement for albumin and EDTA seems to be characteristic of insect particles (mosquito, housefly), since guinea-pig liver mitochondria catalysed the exchange reaction in their absence (Table 4).

Effect of particle concentration

The ATP–^{32}P exchange was found to increase with the particle concentration up to a limit of a concentration equivalent to 0·3 mg. of N/ml. of assay medium. No splitting of ATP occurred at particle concentrations below 0·1 mg. of N/ml. of assay medium (Fig. 1).

Kinetics

The kinetics of the ATP–^{32}P exchange and of the hydrolysis of ATP are compared in Fig. 2. It can be seen that the splitting of ATP ceases after 10 min., whereas the exchange reaction proceeds at a gradually diminishing rate. It should be noted that after 30 min., when the exchange had practically come to an end and about 90% of the ATP was still unhydrolysed, the radioactivity of the phosphate groups in the nucleotide fraction was only approx. 10% of the value calculated for a state of equilibrium between the terminal phosphate of ATP and the added ^{32}P.

When the respiratory particles were pre-incubated for 20 min. at 28° with the assay medium, but the ATP was added only after the pre-incubation, the incorporation of ^{32}P into the nucleotide proceeded at the same rate as under the usual conditions of the reaction. Hence the cessation of the exchange was not due to an inactivation of the particles during the incubation. Accumulation of an inhibitory substance in the medium was indicated by the finding that when the respiratory particles, incubated with the complete reaction mixture, were separated after 10 min. by centrifuging and fresh particles were added to the supernatant no further exchange was observed (Fig. 2).

Table 4. *Requirement for albumin and ethylenediaminetetra-acetic acid of respiratory particles from different sources*

Respiratory particles were obtained as described under Methods, and were added in a concentration equivalent to 0·2 mg. of N/ml. of assay medium. The composition of the assay medium and other conditions of experiment were as described in Table 1, except that albumin and EDTA were added as indicated below.

Source of respiratory particles	Additions to assay medium: Albumin (%, w/v)	Additions to assay medium: EDTA (mM)	ΔP	Total counts in nucleotides/min.
Mosquito	1	1·0	1·4	3340
Housefly	0	0	1·1	120
	0	1·0	0·7	300
	1	0	1·2	200
	1	1·0	0·8	2800
Guinea-pig liver	0	0	0·0	5230
	1	1·0	0·0	5180

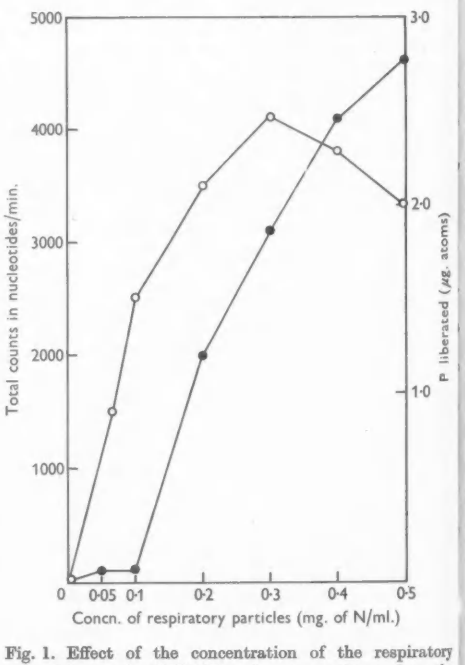

Fig. 1. Effect of the concentration of the respiratory particles on the ATP–^{32}P-exchange reaction and on the ATPase activity. ○, Total counts in nucleotides/min.; ●, μg. atoms of P liberated. Conditions of reaction were as described in Table 1. Incubation was for 10 min. at 28°.

Effect of pH

Exchange between ATP and ^{32}P was observed over a wide range of pH values with a broad peak in the range pH 7·5–8·0. No similar peak was apparent in ATPase activity. The rate of hydrolysis of ATP increased with a rise in the pH (Fig. 3).

Effect of nucleotides

Exchange between ^{32}P and nucleotides occurred at maximum velocity with ATP as the substrate. The rate of incorporation of ^{32}P into adenosine diphosphate (ADP) was approx. 30% of the maximum rate (probably as a result of myokinase activity). Inosine triphosphate (ITP) and adenosine monophosphate (AMP) did not become labelled under the conditions of the experiment. Addition of ADP or ITP inhibited significantly the incorporation of ^{32}P into ATP, whereas AMP was not inhibitory (Table 5).

The nucleotide specificity of the 'ATPase' differed from the exchange system in its effect on ITP. The rate of hydrolysis of ITP was even more rapid than that of ATP. ADP was split slowly and AMP not at all. ADP slightly decreased the rate of hydrolysis of ATP, when it was added in an equimolar amount (Table 5).

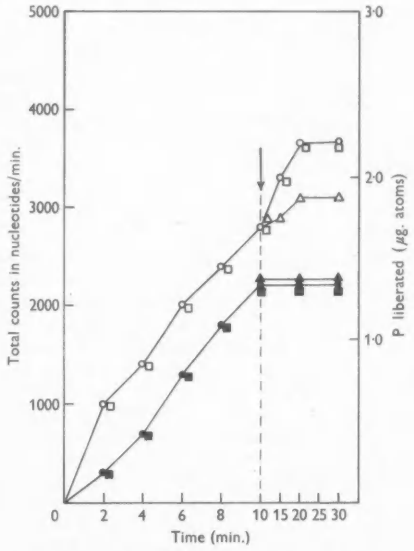

Fig. 2. Kinetics of the ATP–^{32}P exchange and the hydrolysis of ATP. ○, ●, Standard particle preparation; □, ■, particles pre-incubated with the assay medium for 20 min. at 28° in the absence of ATP; △, ▲, at the time indicated by the arrow, particles in experiment ○, ● were replaced by an equal amount of fresh respiratory particles. Other conditions of the reaction were as described in Table 1. Open points (○, □, △) total counts in nucleotides/min.; filled points (●, ■, ▲): μg. atoms of P liberated.

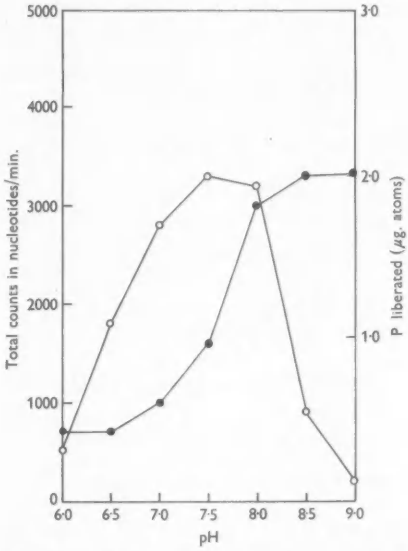

Fig. 3. Effect of pH on ATP–^{32}P exchange and on the ATPase activity. ○, Total counts in nucleotides/min.; ●, μg. atoms of P liberated. Conditions of experiment were as described in Table 1.

Table 5. *Effect of nucleotides*

Particle preparation was isolated and washed as described under Methods. Composition of assay medium, with the exception of the nucleotide, and the conditions of reaction were as described in Table 1.

Nucleotide added	Concn. (mM)	ΔP	Total counts in nucleotides/min.
None	—	0·0	15
ATP	7·0	1·2	3080
ADP	7·0	0·6	910
AMP	7·0	0·0	18
ITP	7·0	1·6	60
In the presence of 7 mM-ATP			
ADP	2·0	1·4	2710
ADP	4·0	1·0	2020
ADP	7·0	1·0	1480
AMP	7·0	1·2	2950
ITP	2·0	1·2	3110
ITP	4·0	1·8	2600
ITP	7·0	2·4	1340

Effect of respiratory inhibitors

A number of substances which inhibit the oxidation of various substrates of the citric acid cycle by mosquito respiratory particles (Gonda *et al.* 1957) were tested for their effect on the ATP–^{32}P-exchange and ATPase activity. Potassium cyanide, sodium azide and *p*-chloromercuribenzoic acid, which were found to inhibit the oxidation of both α-oxoglutarate and succinate, also inhibited the exchange reaction to a similar extent. Arsenite and fluoropyruvate, which were found to inhibit only the oxidation of α-oxoglutarate, were without effect on the ATP–^{32}P exchange (Table 6).

Effect of 2:4-*dinitrophenol*

2:4-Dinitrophenol (DNP), which is well known as an uncoupler of oxidative phosphorylation in mammalian as well as in insect respiratory particles, inhibited the ATP–^{32}P exchange to an extent of 60% at a concentration of 50 μM. Inhibition of the exchange was, however, not paralleled by a strong acceleration of ATPase activity as it is known to occur in liver mitochondria (Boyer *et al.* 1956). Magnesium-stimulated ATPase activity was also unaffected by DNP (Table 7).

Table 6. *Effect of respiratory inhibitors*

Respiratory particles were isolated and washed as described under Methods. The composition of the assay medium and other conditions of the reaction were as described in Table 1.

Inhibitor	Concn. (mM)	ΔP	Total counts in nucleotides/ min.
None	—	2·4	3400
KCN	0·1	2·4	2208
	1·0	2·0	1510
NaN_3	1·0	0·0	1470
	10·0	0·1	30
p-Chloromercuribenzoate	0·01	2·5	3100
	0·1	2·4	120
Arsenite	0·1	2·4	3200
	1·0	2·4	2950
Fluoropyruvate	0·1	2·3	3150
	0·6	2·4	3510

Table 7. *Effect of* 2:4-*dinitrophenol*

Respiratory particles were isolated and washed as described under Methods. The composition of the assay medium and the conditions of the reaction were as given in Table 1.

Concn. of DNP (mM)	Concn. of Mg^{2+} ions (mM)	ΔP	Total counts in nucleotides/ min.
None	—	1·4	2995
0·01	—	1·4	3015
0·03	—	1·4	2820
0·05	—	1·6	1250
0·10	—	2·3	210
—	1·0	3·2	2700
0·10	1·0	3·1	70

Effect of DDT

It has previously been shown that the effect of DDT on oxidative phosphorylation in mosquito particles resembles in many respects that of DNP (Gonda *et al.* 1957). It is seen from Table 8 that the two substances have a similar effect on the exchange and ATPase reactions. DDT at a concentration of 50 μM inhibited significantly the exchange reaction but had little effect on the rate of hydrolysis of ATP. In these experiments DDT was added to the reaction mixture either dissolved in a water-soluble solvent such as ethanol or propanol, or in the form of an emulsion in paraffin oil. Since the detergents tested were deleterious for the enzyme system, albumin was used as a stabilizer of the DDT suspension. The albumin added increased also the stability of the paraffin oil emulsion. A medium containing 50 μM-DDT showed no visible turbidity and became opalescent only when the concentration was increased over and above this value. DDT was completely ineffective in a paraffin oil emulsion. It should be noted that the solvents used (ethanol, propanol) had also some inhibitory effect on the exchange reaction. Guinea-pig liver mitochondria were much less sensitive to DDT than the respiratory particles obtained from the mosquito or the housefly (Table 8).

DISCUSSION

The enzyme system which catalyses the exchange between ^{32}P and ATP in respiratory particles obtained from the mosquito resembles its counterpart in the mammalian mitochondria in many respects. As in mitochondria and submitochondrial preparations from the liver (Cooper & Lehninger, 1957), exchange takes place only with ATP as the substrate, and not with ITP. ADP is inhibitory, and both preparations are sensitive to DNP. Although no endogenous respiration could be demonstrated with the conventional manometric technique, a correlation exists between the effect of respiratory inhibitors on the exchange on the one hand and on the oxidation of succinate on the other.

The reason for the decline in the rate of incorporation of ^{32}P into ATP, before the isotopic equilibrium between the terminal phosphate of the latter and the labelled inorganic phosphate was reached, is not clear. The experimental data are consistent with the view that, during the time of incubation, an inhibitor is formed in the presence of ATP. Although ADP, the product of the hydrolysis of ATP, was indeed found to inhibit the

Table 8. *Effect of* 1:1:1-*trichloro*-2:2-*di*-(p-*chlorophenyl*)*ethane*

Particles were obtained as described under Methods and were added in a concentration equivalent to 0·2 mg. of N/ml. of assay medium. Composition of the assay medium and other conditions of the experiment were as described in Table 1. DDT was added to the reaction mixture dissolved in the solvents indicated.

Source of the particles	Solvent 1% (w/v)	DDT (μM)	ΔP	Total counts in nucleotides/min.
Mosquito	None	None	1·8	3085
	Ethanol	None	1·8	2400
		50	1·8	1900
		100	1·7	1470
		200	2·3	89
	Propanol	None	1·7	1907
		50	1·8	1150
		100	1·9	820
		200	2·2	70
	Paraffin oil	None	1·8	3120
		100	1·8	3200
		400	1·9	3020
Housefly	None	None	0·7	2515
	Propanol	None	0·9	1845
		100	1·2	870
Guinea-pig liver	None	None	0·0	5850
	Propanol	None	0·2	4870
		100	0·2	4550
		200	0·4	3900

exchange, the amount of ADP which could be present after 30 min. of incubation in the reaction mixture is too small to account for the cessation of the exchange.

The addition of serum albumin to preparations of insect sarcosomes has been shown to maintain phosphorylation (Sacktor, 1954; Lewis & Slater, 1954), and to restore phosphorylative activity in mouse-liver mitochondria that were inactivated completely by ageing (Pullman & Racker, 1956). Polis & Shmukler (1954) isolated a haemoprotein which is released from aged mitochondria and which uncouples phosphorylation. This haemoprotein ('mitochrome') is strongly bound by serum albumin. The results of the present investigation can be understood on the basis of the assumption that albumin binds the substance which inhibits ATP–^{32}P exchange. This is indicated by the finding that the presence of albumin was not found to be obligatory throughout the incubation period, provided that it was added to the washing medium during the isolation procedure. Albumin exerted its effect on the exchange, however, only when EDTA was also present.

The ATPase of the mosquito respiratory particles resembles also the ATPase of liver mitochondria. The relative rates of the hydrolysis of different nucleotides (ATP $\cong$ ITP $\gg$ ADP) are similar (Kielley & Kielley, 1951). The lack of proportionality between enzymic activity and particle concentration at low tissue concentrations observed in the mosquito particles has been described also in liver mitochondria (Potter & Recknagel, 1951). The most conspicuous difference between the ATPase of liver mitochondria and that of the mosquito particles is that the acceleration of ATPase and the inhibition of the exchange reaction by DNP are not as closely related in insect sarcosomes as in liver mitochondria (Boyer *et al.* 1956).

The inhibition of the ATP–^{32}P exchange by DDT is of interest in view of the observed great quantitative difference between the insect and mammalian respiratory particles in the extent of inhibition (more than 50% inhibition in insect particles and less than 10% in mammalian liver mitochondria by 0·1 mM-DDT). It should be noted, however, that a statement about the 'concentration' of DDT is ambiguous and might be misleading. DDT is not in a true solution in the system and is divided between at least three phases: the oily suspension, an aqueous phase and the respiratory particles. An equilibrium unfavourable for the particles may explain the lack of effect of DDT in a paraffin oil emulsion. Experiments are now in progress to relate the inhibitory effect of DDT and some of its toxic and non-toxic derivatives on phosphorylative processes to the true concentration of these substances in the respiratory particles.

SUMMARY

1. Adenosine triphosphatase and the enzyme system catalysing the incorporation of ^{32}P into adenosine triphosphate have been studied in

respiratory particles prepared from the mosquito *Aedes aegypti* L.

2. Addition of ethylenediaminetetra-acetic acid to the isolation medium and of ethylenediaminetetra-acetic acid and albumin either to the washing liquid or to the assay medium has been found obligatory for the exchange reaction.

3. The effect of nucleotides, respiratory inhibitors and inhibitors of oxidative phosphorylation on the exchange reaction and on the adenosine triphosphate activity has been investigated.

4. Quantitative differences exist between the response of insect and mammalian respiratory particles to 1:1:1-trichloro-2:2-di-(*p*-chlorophenyl)-ethane (DDT). 0·1 mM-DDT inhibits the exchange reaction by more than 50% in insect sarcosomes whereas the inhibition in mammalian liver mitochondria is less than 10%.

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The Biosynthesis of Porphyrins from Porphobilinogen by *Rhodopseudomonas spheroides*

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In a comprehensive review (van Niel, 1944) of the non-sulphur purple and brown bacteria or Athiorhodaceae there was described the production of a diffusible red pigment from growing cells. Some properties of this pigment were described, including some of its absorption characteristics and solubility in organic solvents. In the light of subsequent studies it seems clear that the pigment described was a porphyrin derivative. Since members of the Athiorhodaceae are photosynthetic and thus contain bacteriochlorophyll and, further, since they have been shown to be rich in haem derivatives (Elsden, 1954) they possess potent enzyme systems capable of synthesizing the porphyrin nucleus. The studies of Lascelles (1955, 1956) with whole-cell suspensions of *Rhodopseudomonas spheroides*, a typical member of the group of the Athiorhodaceae, have indeed shown that these organisms can bring about a synthesis of free porphyrins and bacteriochlorophyll from glycine together with certain intermediates of the tricarboxylic acid cycle.

Lascelles (1955, 1956) found that δ-aminolaevulic acid was converted into porphyrins by cell suspensions of *R. spheroides* and during the initial stages of the reaction porphobilinogen diffused out of the cells. However, when porphobilinogen was added to washed cell suspensions of *R. spheroides* it was not converted into porphyrins, although it is a porphyrin precursor in other systems. (For a review of the interrelationships of δ-aminolaevulic acid, porphobilinogen and porphyrins see Rimington, 1957.) Bogorad & Granick (1953) have shown that porphobilinogen is converted into porphyrins by suspensions of *Chlorella vulgaris* provided that they have been previously subjected to freezing and thawing. This suggests that porphobilinogen is not permeable to intact whole cells; a similar observation was made with avian erythrocytes (Dresel & Falk, 1956*a*).

The findings of Kikuchi, Shemin & Bachmann (1958) and of Gibson (1958), establishing the biosynthesis of δ-aminolaevulic acid from glycine and

succinyl-coenzyme A, and the requirements for pyridoxal phosphate at this stage, in preparations of *R. spheroides*, further confirm the important role of δ-aminolaevulic acid in porphyrin biosynthesis by this bacterium. δ-Aminolaevulic acid dehydrase, which converts δ-aminolaevulic acid into porphobilinogen, is widely distributed in animals, plants and micro-organisms (Gibson, Neuberger & Scott, 1955), and is presumably present in *R. spheroides*, accounting for the production of porphobilinogen by washed cell suspensions when incubated with δ-aminolaevulic acid.

The object of this work was to study in more detail the steps involved in the biosynthesis of porphyrins from porphobilinogen. The formation of uroporphyrin from porphobilinogen has been studied with *Chlorella* (Bogorad & Granick, 1953), with spinach preparations by Bogorad (1955, 1957*a*, *b*) and with chicken-blood preparations (Dresel & Falk, 1956*a*, *b*; Lockwood & Rimington, 1957). Preliminary reports of these studies have appeared elsewhere (Heath & Hoare, 1958; Hoare & Heath, 1958*a*, *b*).

EXPERIMENTAL

Organism. The strain of *R. spheroides* used was obtained from Dr June Lascelles and came originally from the collection of Professor C. B. van Niel. It was maintained as described by Lascelles (1956).

Growth and harvesting of organism. The organism was grown on the 'medium S' described by Lascelles (1956) but 0·2% (w/v) of yeast extract was substituted for the pure vitamins. Cells were grown semi-anaerobically in the light in a 1 l. Roux bottle filled to the shoulder, plugged with cotton wool and illuminated by a single 40w tungsten lamp at a distance of 6–9 in.; this maintained the culture at a temperature of 30° (±2°). The cells were harvested towards the end of the logarithmic phase of growth (usually 72 hr.) by centrifuging at 12 000 *g* at 1° for 10 min. The packed cells were washed once with 0·9% NaCl soln. The yield was 8–9 g. wet wt. (equivalent to 1·4–1·6 g. dry wt.). The packed cells were stored at −15° unless used directly.

Growth of cells aerobically in the dark. The same medium was used as in the semi-anaerobic, light-grown cultures; 100 ml. of medium contained in 500 ml. Erlenmeyer flasks was inoculated with a cell suspension, the flasks were covered with dark paper and were clamped to a rotary shaker at room temperature (20°). The cells were harvested after reaching maximum growth in 3 or 4 days.

Buffer solutions. McIlvaine's citric acid–Na_2HPO_4 buffers, made up in de-ionized water, were used to cover the range pH 3·0–8·0 (*Documenta Geigy Scientific Tables*, 1956). Porphobilinogen (PBG), uroporphyrin I and III and coproporphyrin I and III were generously provided by Professor C. Rimington, F.R.S. Solutions were made up in a 1:10 (v/v) dilution of buffer, pH 6·5, and were stored at −15°.

Treatment of cells for enzyme experiments. (*a*) Frozen and thawed cell preparations. Cells were suspended in buffer (250 mg. wet wt. of cells/ml., equivalent to 45 mg. dry wt./ml.) and were completely frozen by immersion in a glycol bath at −15°. The cell suspensions were allowed to thaw at room temperature. This procedure was repeated twice.

(*b*) Preparation of cell-free extracts by grinding with alumina. The method used was that described by McIlwain (1948). Frozen packed cells (2·5 g.) were mixed with an equal weight of Microid Polishing Alumina, grade 3/50 (Griffin and George Ltd., London) in a glass mortar. All materials were previously cooled to −15°. Grinding was continued with small additions of cooled buffer at pH 6·5 until a sticky paste was obtained. A total of 10 ml. of buffer was finally added and the suspension was centrifuged at 12 000 *g* for 15 min. at 1°. This produced a clear dark-brown supernatant which was removed with a Pasteur pipette.

(*c*) Preparation of acetone-dried powders. The packed cells were suspended in water and a large excess (at least 10 vol.) of acetone, cooled to −15°, was added. After standing for 30 min. at −15° the precipitate which had formed was filtered off, washed with cold acetone and dried in a vacuum desiccator over silica gel. This produced a pale grey–green powder which was stored at −15°.

Incubation conditions. Reaction mixtures of total volume 0·5 ml., comprising 0·4 ml. of the test preparation in buffer and 0·1 ml. of PBG soln. (1 mg. of the free base monohydrate/ml.), were incubated at 37° in glass centrifuge tubes (10 cm. × 1 cm.), plugged with cotton wool.

Estimation of porphyrins. The reaction was stopped by adding 5 ml. of ethyl acetate–acetic acid mixture (3:1, v/v) and the mixture was left for 1 hr. in the dark. After centrifuging, the clear supernatant was decanted into a 60 ml. separating funnel, the residue was resuspended in a further 5 ml. of ethyl acetate–acetic acid and, after centrifuging, the two supernatants were combined. Uroporphyrin was then extracted with 3 × 5 ml. of a saturated solution of sodium acetate followed by 5 ml. of 3% (w/v) sodium acetate soln. Each extract was re-extracted in a second separating funnel with 10 ml. of ethyl acetate. When coproporphyrin was formed some was extracted into sodium acetate together with uroporphyrin, but the second ethyl acetate extraction retained the coproporphyrin. Uroporphyrin was extracted from the combined sodium acetate extracts by the *cyclo*hexanone procedure (Dresel & Falk, 1956*a*) and was estimated in 5% (w/v) HCl. The combined ethyl acetate washings and the original ethyl acetate–acetic acid solutions were combined and extracted with 3 × 5 ml. of 15% (w/v) HCl. The combined acid extracts were adjusted to pH 4 with solid sodium acetate and the coproporphyrin was extracted into 20 ml. of ether. The aqueous phase was extracted in a second funnel with a further 20 ml. of ether. The two ether solutions were then washed in succession with saturated sodium acetate, 3% sodium acetate and water. The combined ether phases were then extracted to completion with 10% (w/v) HCl. Porphyrin concentrations in the HCl solutions were determined at the Soret maxima in a Unicam SP. 500 spectrophotometer with the correction factors of Rimington & Sveinsson (1950).

Estimation of porphobilinogen. The reaction was stopped by adding 1·5 ml. of 6·7% (w/v) trichloroacetic acid. After centrifuging, suitable portions of the clear supernatant were diluted to 2 ml. with 5% (w/v) trichloroacetic acid

and 2 ml. of a modified Ehrlich reagent (15 g. of *p*-dimethylaminobenzaldehyde dissolved in 64 ml. of 10N-HCl and 195 ml. of acetic acid) was added. The maximum extinction at 555 mμ, usually attained in 1–5 min., was determined in a Unicam spectrophotometer. The amount of PBG present was calculated from a standard curve. ($E^{1\,cm.}_{555\,m\mu}/0{\cdot}114 = \mu g.$ of PBG monohydrate/ml. of 5% trichloroacetic acid test solution).

Chromatography of porphyrins. Identification of porphyrins and porphyrin isomers was carried out by the method of Falk & Benson (1953) and of Falk, Dresel, Benson & Knight (1956).

Separation of uroporphyrin I- and uroporphyrin III-forming enzymes. Cells grown semi-anaerobically in the light were harvested by centrifuging and washed once with 0·9% NaCl soln. The packed cells (6 g. wet wt.) were suspended in 10 ml. of water and frozen in a solid CO_2–acetone bath for 10 min. and then thawed at 15°; this procedure was twice repeated and, after centrifuging for 10 min. at 12 000 *g*, the supernatant was removed. The cell residue was resuspended in water (10 ml.) and the whole procedure was repeated a further three times. The four supernatant fractions (total volume 40 ml.) thus obtained were combined, cooled to 1° and acetone (400 ml.), previously cooled to −15°, was added. After standing at −15° for 30 min. the precipitate (110 mg.), which contained the enzyme(s) converting PBG into uroporphyrin I, was collected by centrifuging.

The final cell residue was suspended in water (10 ml.) and treated with cold acetone (200 ml.) to yield a green powder (240 mg.) containing the enzyme(s) converting PBG into uroporphyrin III.

RESULTS

Whole-cell suspensions. Cells harvested from cultures grown semi-anaerobically in the light, and washed and suspended in buffer to a concentration of 12 mg. dry wt./0·5 ml., on incubation at pH 6·2, 6·6, 6·8, 7·0 and 7·2 for 16 hr. at 37° with PBG (50 μg.) formed less than 2 μg. of uroporphyrin and no coproporphyrin. The small amounts of uroporphyrin formed were of the same order as that formed non-enzymically.

Frozen and thawed cell suspensions. After cell suspensions in buffer had been frozen and thawed three times successively, considerable amounts of porphyrins were formed on incubation with PBG for 16 hr. at 37°. At pH 6·1–6·8 the total porphyrin formed accounted for about 50% of the PBG added. The effect of pH on porphyrin formation under these conditions is shown in Table 1. The uroporphyrin figures are not corrected for the non-enzymic conversion of PBG into uroporphyrin (Cookson & Rimington, 1954). Since maximum coproporphyrin formation occurred at pH 6·5 under the above conditions, this pH was used for the further study of porphyrin formation from PBG.

Time course of the reaction with frozen and thawed cell suspensions. Washed cells equivalent to 18 mg. dry wt. were suspended in 0·4 ml. of buffer, pH 6·5, and the suspension was frozen and thawed three times and incubated at 37° with 0·1 ml. of PBG soln. (100 μg.). Tubes were set up in duplicate for each incubation time, one tube for the estimation of porphyrins and the other for PBG. The course of the reaction is illustrated in Fig. 1. Such preparations formed virtually only coproporphyrin; the small amounts of uroporphyrin could have been formed non-enzymically. Coproporphyrin production reached a maximum after incubation for 4 hr. Disappearance of PBG was very rapid, and was complete after 6 hr.; only half could be accounted for as porphyrin. Controls in which PBG solutions were incubated in buffer for different times without enzyme showed appreciable disappearance of Ehrlich-reacting material with only very slight formation of uroporphyrin.

Table 1. *Effect of* pH *on porphyrin formation by frozen and thawed suspensions of cells incubated with porphobilinogen*

Cells (25 mg. dry wt./tube) in 0·4 ml. of buffer with 0·1 ml. of PBG soln. (100 μg.). Incubation: 16 hr. at 37°.

	Porphyrin (μg.)		
pH	Uro-	Copro-	Total
4·2	1	2	3
4·6	1	7	8
5·2	3	15	18
5·6	5	22	27
6·1	4	34	38
6·3	5	34	39
6·5	2	36	38
6·8	0	33	39
7·2	3	34	37
7·5	10	31	41
8·0	11	32	43

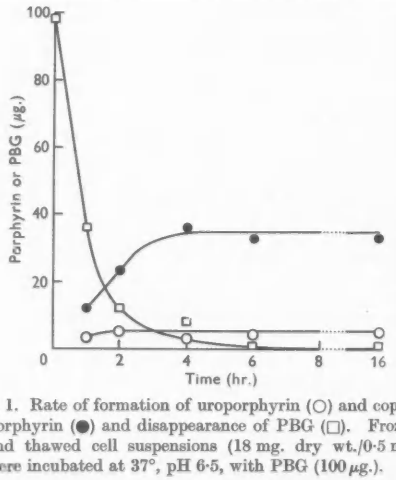

Fig. 1. Rate of formation of uroporphyrin (○) and coproporphyrin (●) and disappearance of PBG (□). Frozen and thawed cell suspensions (18 mg. dry wt./0·5 ml.) were incubated at 37°, pH 6·5, with PBG (100 μg.).

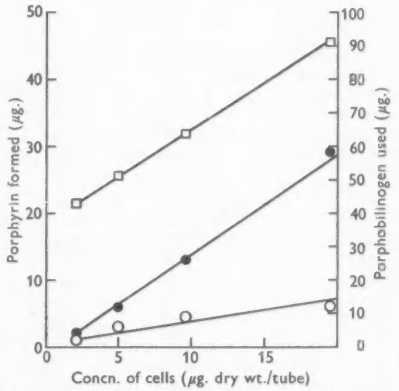

Fig. 2. Effect of cell concentration on the formation of uroporphyrin (○) and coproporphyrin (●) and on the disappearance of PBG (□). Frozen and thawed cell suspensions were incubated for 4 hr. at 37°, pH 6·5, with PBG (100 μg.).

Table 2. *Recovery of added porphyrins after incubation for 4 hr. either in buffer, pH 6·5, or in the presence of frozen and thawed cell suspensions (18 mg. dry wt./tube)*

Porphyrin added	Wt. (μg.)	Porphyrin recovered (μg.)			
		Buffer		Cell suspension	
		Copro-	Uro-	Copro-	Uro-
Uro- I	44	0	36	0	30
Uro- III	46	0	39	0	33
Copro- I	45	38	0	37	0
Copro- III	44	39	0	38	0

Table 3. *Separation of the frozen and thawed cell porphyrin-forming systems*

Cells (45 mg. dry wt./ml.) were frozen and thawed three times and separated by centrifuging into supernatant and residual cells. Incubation: 16 hr. at 37° with 100 μg. of PBG.

	Porphyrin (μg.)		
	Copro-	Uro-	Total
Whole cells	38	7	45
Supernatant	2	29	31
Residual cells	22	13	35
Supernatant plus residual cells	41	6	47

Frozen cell suspensions in 0·9 % NaCl soln., kept at −15° for 2 weeks, showed no loss of enzyme activity when assayed for 16 hr.; 39 μg. of total porphyrin (36 μg. of coproporphyrin and 3 μg. of uroporphyrin) was formed from 100 μg. of PBG. After storage for 2 months the total porphyrin formed was 31 μg. (18 μg. of coproporphyrin and 13 μg. of uroporphyrin).

Over an incubation period of 4 hr. and a range of cell concentrations from 2 to 20 mg. dry wt./tube, the amounts of porphyrin formed and of PBG used were proportional to the concentration of cells (Fig. 2).

Coproporphyrin was not formed when uroporphyrin I or uroporphyrin III was incubated with frozen and thawed cell suspensions. Control tubes in which uroporphyrins I and III and coproporphyrins I and III were similarly incubated in buffer alone showed recoveries of 80–90 % (Table 2).

Separation of frozen and thawed cell suspensions into enzyme systems forming uroporphyrins I and III respectively. When a frozen and thawed cell suspension was centrifuged, it was found that the clear supernatant formed uroporphyrin from PBG. The residual cells, on resuspending in buffer, formed less coproporphyrin and more uroporphyrin than the original frozen and thawed preparation. However, when the residual cells were resuspended in the supernatant, coproporphyrin production was comparable with that found with the original frozen and thawed cell suspension (Table 3). If the freezing and thawing procedure and subsequent separation was repeated several times, the ability of the cell residue to synthesize coproporphyrin progressively diminished and at the same time the formation of uroporphyrin correspondingly increased (Table 4). The total amount of porphyrin formed remained fairly constant. The supernatant, however, formed very little coproporphyrin from PBG. Reconstitution of the above system by adding a boiled supernatant to the extracted cell residue gave an increase in coproporphyrin production and a decrease in uroporphyrin (Table 5). The boiled supernatant alone formed only small amounts of uroporphyrin non-enzymically. The uroporphyrins and coproporphyrins isolated from these fractions were examined chromatographically for isomer type. The frozen and thawed cells formed mainly coproporphyrin III with traces of coproporphyrin I, the uroporphyrin formed by the cell residue after removal of the supernatant was mainly type III, whereas the supernatant itself formed uroporphyrin type I.

Cell-free extracts. Cell-free extracts were prepared by grinding with alumina and extracting in buffer. The resulting clear-yellow–brown extracts converted added PBG into both uroporphyrin and coproporphyrin; the total and the relative amounts of the porphyrins depended on both the time of incubation and the enzyme concentration. The course of the reaction is shown (Fig. 3) for a concentration of extract equivalent to 18 mg. dry wt. of whole cells; the system is thus comparable with that of the frozen and thawed cells illustrated in

Table 4. *Effect of repeated extraction of cells by freezing and thawing in buffer*

A volume (30 ml.) of cell suspension (45 mg. dry wt./ml.) in buffer, pH 6·5, was frozen and thawed three times and 0·4 ml. (equivalent to 18 mg. dry wt.) was used for assay of 'original cells'; cell suspension was centrifuged and 0·4 ml. of supernatant assayed. Cell residue was resuspended in buffer to a cell concentration of 45 mg. dry wt./ml. and again frozen and thawed three times; 0·4 ml. of suspension was used for assay of 'first-treatment' cells; cell suspension was centrifuged and 0·4 ml. of the corresponding supernatant assayed. The above process was repeated on the cell residue for the second and third treatments. Incubations: 16 hr. at 37°, pH 6·5, with 100 μg. of PBG.

	Porphyrin (μg.)					
	Cells			Supernatant		
	Copro-	Uro-	Total	Copro-	Uro-	Total
Original cells	37	3	40	2	20	22
First treatment	27	6	33	0	15	15
Second treatment	13	25	38	1	30	31
Third treatment	6	38	44	2	21	23

Table 5. *Porphyrin formation by frozen and thawed cell suspensions partially reconstituted with boiled supernatant*

Cell suspension in water (45 mg. dry wt./ml.) was frozen and thawed three times and centrifuged; cell residue and supernatant (equivalent to 18 mg. dry wt. of original cells) were incubated for 16 hr. at 37°, pH 6·5, with 100 μg. of PBG.

	Porphyrin formed (μg.)		
Enzyme system	Uro-	Copro-	Total
Cell residue	11	27	38
Boiled supernatant	9	0	9
Residue + boiled supernatant	5	35	40
Residue + supernatant	5	38	43

Fig. 1. By contrast, the cell-free extracts formed more uroporphyrin initially, reaching a maximum in 2 hr. After incubation for 16 hr. at 37°, pH 6·5, formation of coproporphyrin was approximately proportional to the concentration of cell-free extract. Uroporphyrin formation, however, deviated considerably from such proportionality: at high enzyme concentrations more coproporphyrin than uroporphyrin was formed; the reverse was true at low concentrations (Table 6). Magnesium ions (mM) had no effect on the nature or quantity of porphyrins formed. Chromatographic examination of the reaction products showed the 'uroporphyrin' fraction to consist of a mixture of the I- and III-series isomers, together with small amounts of penta-, hexa- or hepta-carboxylic porphyrin; such intermediate carboxylic porphyrins were also observed in the other systems.

Compared with the frozen and thawed cell preparations, the cell-free extracts were relatively unstable. A fresh preparation formed 13 μg. of coproporphyrin and 24 μg. of uroporphyrin on incubation for 2 hr. with 100 μg. of PBG, and after storage for 3 days at $-15°$ it then formed only 5 μg. of coproporphyrin and 11 μg. of uroporphyrin.

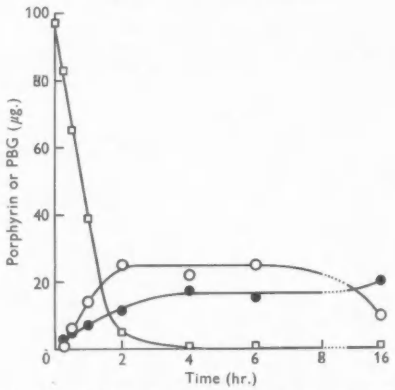

Fig. 3. Rate of formation of uroporphyrin (○) and coproporphyrin (●) and disappearance of PBG (□). Cell-free extract of *R. spheroides* (0·4 ml.; 2·9 mg. dry wt., equivalent to 18 mg. dry wt. of whole cells) was incubated at 37°, pH 6·5, with 0·1 ml. (100 μg.) of PBG soln.

Table 6. *Porphyrin formation by cell-free extracts of* Rhodopseudomonas spheroides: *effect of enzyme concentration*

Reaction mixture (total volume 0·5 ml.) contained cell-free extract at pH 6·5 and 100 μg. of PBG and was incubated for 16 hr. at 37°.

Concn. of enzyme (mg. dry wt./tube)	Porphyrin formed (μg.)		
	Uro-	Copro-	Total
2·4	11	23	34
0·6	37	6	43
0·24	27	2	29
0·06	11	0	11

Enzymic activity of acetone-dried powders. Acetone-dried powders of semi-anaerobic, light-grown cells appeared to be more active than either frozen and thawed cell preparations or cell-free extracts in

Table 7. *Porphyrin formation by acetone-dried powders of (a) semi-anaerobic, light-grown and (b) aerobic, dark-grown cells of* Rhodopseudomonas spheroides: *effect of enzyme concentration*

Reaction mixture (total volume 0·5 ml.) contained the acetone-dried powder in buffer, pH 6·5, and 100 μg. of PBG and was incubated for 4 hr. at 37°.

Acetone-dried powder (mg. dry wt./tube)	Porphyrins formed (μg.)		
	Uro-	Copro-	Total
	(*a*)		
18·0	4	30	34
4·5	33	4	37
1·8	24	1	25
	(*b*)		
18·0	18	18	36
9·0	29	8	37
4·5	24	2	26
1·8	7	0	7

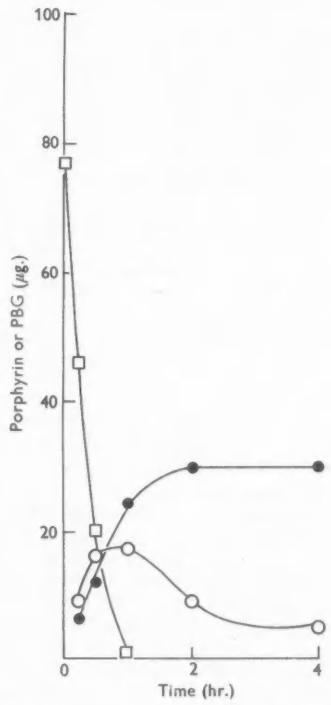

Fig. 4. Rate of formation of uroporphyrin (○) and coproporphyrin (●) and disappearance of PBG (□). Acetone-dried powder of *R. spheroides* (18 mg.) in 0·4 ml. of buffer, pH 6·5, was incubated at 37° with 0·1 ml. (80 μg.) of PBG soln.

forming porphyrins from PBG. As with cell-free extracts, the nature and relative proportions of the porphyrins produced depended on the enzyme concentration as well as on the time of incubation. Table 7 shows that over an incubation period of 4 hr. coproporphyrin production exceeds uroporphyrin production with high concentrations of enzymes, but uroporphyrin predominates if low concentrations are employed. The time course for the reaction is illustrated in Fig. 4. The pH optimum for coproporphyrin formation over a short incubation period (75 min.) was 7·0 (Fig. 5).

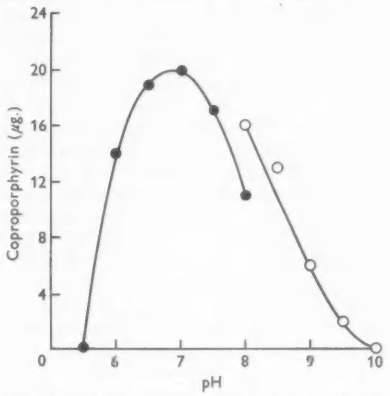

Fig. 5. Effect of pH on coproporphyrin formation from PBG. Acetone-dried powder (20 mg.) in 0·4 ml. of buffer was incubated for 75 min. at 37° with 0·1 ml. (100 μg.) of PBG soln. ●, McIlvaine citric acid–Na_2HPO_4 buffer; ○, borate buffer.

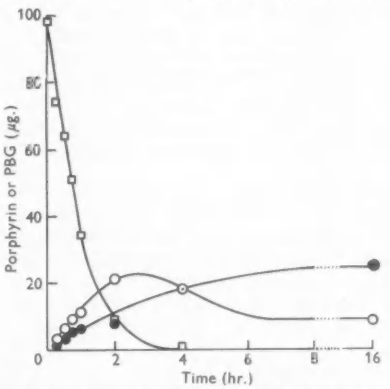

Fig. 6. Rate of formation of uroporphyrin (○) and coproporphyrin (●) and disappearance of PBG (□). Acetone-dried powder of *R. spheroides* grown aerobically in the dark: 18 mg. of powder in 0·4 ml. of buffer, pH 6·5, was incubated at 37° with 0·1 ml. (90 μg.) of PBG soln.

Added uroporphyrins I and III were not decarboxylated by acetone-dried powders even after incubation for 16 hr. at pH 6·5 and 37°.

Acetone-dried powders of aerobic, dark-grown cells also formed porphyrins but were less active (Table 7). A similar effect of enzyme concentration and time of incubation was observed on the relative quantities of uroporphyrin and coproporphyrin formed (Fig. 6). The uroporphyrin formed was the III-series isomer.

Preparation of enzymes converting porphobilinogen into uroporphyrin I and uroporphyrin III respectively. After repeated extraction by freezing and thawing, acetone-dried powders of the extracted cell residue and of the combined supernatants were prepared as described in the Experimental section. The cell-residue fraction converted PBG into uroporphyrin III, whereas the acetone precipitate of the combined supernatants formed uroporphyrin I. A quantity (5 mg.) of the cell-residue acetone-dried powder incubated with PBG (100 μg.) formed 38 μg. of uroporphyrin III in 16 hr., 5 mg. of the acetone-precipitated supernatant fraction formed 33 μg. of uroporphyrin I in 16 hr. Acetone-dried powders of both whole cells and extracted cells after 30 min. preheating at 60–65° converted PBG into uroporphyrin I. Preheated preparation (5 mg. of acetone-dried powder of whole cells) formed 27 μg. of uroporphyrin I after incubation with PBG for 16 hr.

DISCUSSION

The fact that PBG gives rise to porphyrins on incubation with frozen and thawed cell suspensions, but not with washed cell suspensions of *R. spheroides*, could be due to the failure of PBG to enter the intact bacterial cell. The passage of PBG through the intact cell wall appears to take place in one direction only, since PBG diffuses out of intact cells when incubated with δ-aminolaevulic acid (Lascelles, 1956). Frozen and thawed cells differ from both cell-free extracts and acetone-dried cells in forming very little uroporphyrin at any stage. This is attributed to the fact that even in the frozen and thawed cell, the rate of penetration of PBG to the enzymes within the cell may still be the limiting step in the overall process of converting PBG into coproporphyrin. The studies on cell-free extracts and acetone-dried powders indicate that uroporphyrin formation proceeds more rapidly than coproporphyrin formation, and so some step preceding uroporphyrin formation must be rate-limiting in frozen and thawed cells, otherwise larger amounts of uroporphyrin would accumulate. The small amounts of uroporphyrin formed by frozen and thawed cells could arise non-enzymically from PBG. The fact that acetone-dried powders are more active than frozen and thawed cells on a dry-weight basis is further evidence that the rate of access of PBG to the intracellular enzymes is limiting in the latter system.

The observation that cell-free extracts on dilution form more uroporphyrin than coproporphyrin could be due to the fact that uroporphyrin-forming activity is much greater than coproporphyrin activity in the whole micro-organism; alternatively the uroporphyrin-forming enzyme may be more readily extracted into solution after grinding with alumina. A further possibility is that coproporphyrin-forming enzyme is partially destroyed by the alumina-grinding and extraction procedure. Certainly the uroporphyrin-forming enzyme system is the more stable, since cell-free extracts on storage rapidly lost their ability to form coproporphyrin without a comparable loss of ability to form uroporphyrin.

The time course for the reaction of PBG with acetone-dried powders suggested that uroporphyrin may be a direct precursor of coproporphyrin. Since uroporphyrins I and III were not converted into coproporphyrin by such preparations, even after incubation for 16 hr., this possibility is ruled out. Some preliminary investigations have demonstrated that uroporphyrinogens I and III are converted in high yield into coproporphyrins I and III respectively by acetone-dried powders of *R. spheroides* (Hoare & Heath, 1958*a*). Bogorad (1957*b*) has demonstrated the formation of porphyrins with less than eight carboxyl groups by *Chlorella* after incubation with uroporphyrinogen III. Bashour (1956) has reported the conversion of uroporphyrinogen I into coproporphyrin and protoporphyrin by dog-liver homogenates.

The exact relationship between the uroporphyrin I- and uroporphyrin III-forming enzyme systems still remains obscure. Investigations by Bogorad & Granick (1953) on *Chlorella* and by Booij & Rimington (1957) and Lockwood & Rimington (1957) on chick haemolysates have shown that enzyme preparations forming uroporphyrin III are heat-labile and, after heating to 50–65° for short periods of time, then form uroporphyrin I from PBG. Recently Granick & Mauzerall (1958) and Mauzerall & Granick (1958) have prepared from rabbit reticulocytes by starch electrophoresis a 'porphobilinogenase' which converted PBG into uroporphyrinogen III, but after preheating it formed uroporphyrinogen I. We have confirmed these findings with *R. spheroides*. The further observation that frozen and thawed cell suspensions of *R. spheroides* were separated by centrifuging into a supernatant fraction forming uroporphyrin I and a residue forming mainly uroporphyrin III indicates that two enzyme systems are involved. These may be analogous to the 'porphobilinogen

deaminase' and 'uroporphyrinogen isomerase' described by Bogorad (1957*a*), in which case it is assumed that 'porphobilinogen deaminase' is partially extracted into solution by freezing and thawing whereas the isomerase together with some of the deaminase remains with the cell residue. It is not considered likely that the uroporphyrin I-forming enzyme in the supernatant fraction of frozen cells is derived from a uroporphyrin III-forming enzyme by modification of the latter as a result of freezing and thawing. The residual cells retain a very active uroporphyrin III-forming enzyme after ten or more successive extractions by freezing and thawing. The uroporphyrin I-forming enzyme concentration in the supernatant is very low, since very little protein was extracted and only small amounts of uroporphyrin I were formed from PBG after short incubation periods.

Further, when repeatedly extracted cells which formed uroporphyrin from PBG were preheated at 60° for 30 min. uroporphyrin I was formed from PBG. The resolution of frozen and thawed cells into enzyme systems forming uroporphyrins I and III could be attributed to a preferential extraction of PBG deaminase forming uroporphyrin I, the residual cells still containing some PBG deaminase but with a more active 'isomerase' so that uroporphyrin III is formed from PBG. Bogorad (1957*a*, *b*; 1958*a*, *b*) has effected a partial purification of PBG deaminase and of uroporphyrinogen isomerase, and it seems clear that uroporphyrin III is formed by the combined action of the two enzymes PBG deaminase and uroporphyrinogen isomerase, the latter having no action on PBG alone. Previously Bogorad & Granick (1953) and Shemin, Russell & Abramsky (1955) postulated a reaction scheme for the formation of uroporphyrin III which involved the formation of branched tripyrromethanes. Several objections to this scheme were raised, on purely chemical grounds, by Bullock, Johnson, Markham & Shaw (1958). These authors have put forward a reaction mechanism, based on a suggestion of Robinson (1955), which involves carbonium ions in an intramolecular process. Whether such a mechanism is involved in the enzymic synthesis of uroporphyrin III is a matter to be investigated.

The separation of a frozen and thawed cell suspension into a cell residue and supernatant fraction also demonstrates that the formation of coproporphyrin from PBG, via the decarboxylation of a uroporphyrinogen, requires the presence of the supernatant solution, since the thoroughly extracted cell residue converts PBG only into uroporphyrin. The partial restoration of coproporphyrin-forming activity by the addition of a boiled supernatant to the extracted residue suggests that a heat-stable cofactor may be required for the decarboxylation step. We have shown that both uroporphyrinogens I and III are precursors of coproporphyrins I and III in *Rhodopseudomonas spheroides*. Mauzerall & Granick (1958) have investigated the substrate specificity of a partially purified uroporphyrinogen decarboxylase from reticulocytes and have shown that all four isomers can be decarboxylated. Bogorad (1958*a*–*c*) has also investigated the enzymic synthesis of porphyrins from porphobilinogen and has studied coproporphyrin formation from uroporphyrinogen.

SUMMARY

1. Frozen and thawed cells of *Rhodopseudomonas spheroides*, but not whole cells, convert porphobilinogen into uroporphyrin and coproporphyrin.

2. Acetone-dried powders of whole cells are stable when stored at $-15°$ and are the most active source of a coproporphyrin III-forming system.

3. The presence of a heat-stable cofactor necessary for coproporphyrin formation has been demonstrated.

4. A uroporphyrin I-forming system has been separated, by freezing and thawing, from a uroporphyrin III-forming system without the destruction of the latter. Stable acetone-dried powders of these two systems have been prepared.

5. The effects of change of pH, time of incubation and concentration of enzymes have been studied both with frozen and thawed cells and acetone-dried powders.

6. Neither uroporphyrin I nor uroporphyrin III is converted into coproporphyrin, either by frozen and thawed cells or by acetone-dried powders.

7. Cell-free extracts have been made by grinding with alumina. These extracts have weak and unstable coproporphyrin-forming activity.

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The Effect of Monoiodoacetate on the Aerobic Metabolism of Ox Retina *in vitro*

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Noell (1951) and Schubert & Bornschein (1951) have shown that, in a number of species, injection of sublethal doses of monoiodoacetate abolishes the retinogram (the change in potential across the retina caused by light) and destroys the visual cells. Noell found that the changes in the retinogram differed from those caused by anoxia and he suggested that the damage was due to inhibition of glycolytic reactions and that oxidative processes were unaffected. This suggestion is supported by the experiments of Graymore & Tansley (1959), in which the anaerobic glycolysis of rat retina fell to 20% of the normal value within 10 min. of injection of iodoacetate, and by Oguchi's (1938) report that respiration of rabbit and frog retina was not inhibited after injection of iodoacetate. Noell (1958) has also reported experiments which confirm Oguchi's finding, although Auricchio (1950) found that addition of iodoacetate *in vitro* did depress oxygen uptake of rabbit retina.

The lack of effect of iodoacetate on oxygen uptake of retina cannot be regarded as established, for respiration in this tissue is dependent on the composition of the incubation medium and is much faster in a bicarbonate than in a phosphate buffer (Laser, 1937; Craig & Beecher, 1943*a*). Krebs–Ringer phosphate solution was used for the experiments reported by Noell (1958), but Oguchi (1938) does not state the composition of the Ringer solution which he used. Auricchio (1950) used a Ringer phosphate solution but with air as the gas phase and so introduced some carbon dioxide into his system. Reports of the effect of iodoacetate on oxygen uptake of other tissues are conflicting. Lundsgaard (1930), Stannard (1937) and Saslow (1937) found that iodoacetate had no effect on carbohydrate oxidation in frog muscle, whereas Meyerhof & Boyland (1931), who also used frog muscle, and Krebs (1931) and Quastel & Wheatley (1932), working with brain and other tissues from a variety of species, observed a decrease either in oxygen uptake or in respiratory quotient. Barker, Shorr & Malam (1939) found that the effect varied with the tissue used (cat and dog); with testis oxygen uptake was always inhibited to a greater extent than glycolysis, but with heart and brain, at concentrations of iodoacetate which inhibited anaerobic glycolysis nearly completely, the respiratory quotient was not depressed and glucose was oxidized, although in a number of experiments the amount of oxygen used was reduced.

In the experiments described below iodoacetate has been added to ox retina incubated in bicarbonate and in phosphate buffer. In bicarbonate there was a pronounced inhibitory effect but in phosphate inhibition was small and only just significant.

METHODS

Ox eyes were brought from the slaughter house on ice, and the retinae dissected and placed in ice-cold, oxygenated Ringer solution. When a sufficient number had been dissected a whole retina, or in some experiments a part of a retina, was placed in a Warburg flask containing the appropriate Ringer solution (see below), gassed with O_2 or a mixture containing $O_2 + CO_2$ (95:5) and incubated at 37° for 40 or 60 min. At the end of this time portions of the medium were removed for estimation of lactic acid (Barker & Summerson, 1941) and glucose (Somogyi, 1945) or pyruvate (Umbreit, Burris & Stauffer, 1945). The remaining contents of the flask were poured into a small weighed centrifuge tube, and the tissue was spun down, dried and weighed. The glycogen content (Good, Kramer & Somogyi, 1933) and the ratio of wet weight to dry weight was determined on a number of retinae before incubation.

Incubation in Ringer phosphate solution. Krebs–Ringer phosphate solution was prepared according to the formula given by Umbreit *et al.* (1945), by mixing 100 ml. of 0·154 M-NaCl, 4 ml. of 0·154 M-KCl, 3 ml. of 0·11 M-$CaCl_2$, 1 ml. of 0·154 M-KH_2PO_4, 1 ml. of 0·154 M-$MgSO_4$ and 12 ml. of 0·1 M-Na_2HPO_4 containing 0·02 N-HCl; glucose or pyruvate and iodoacetate were added as required and the medium was gassed with O_2. In the first series of experiments small pieces of retina (10–20 mg. dry wt.) were incubated in 1·5 ml. of medium for 60 min., but in a second series whole retinae, or larger pieces (30–75 mg. dry wt.) were incubated in 3 ml. of medium for 40 min. only. There was no significant difference in the amount of O_2 used during the first 40 min. of incubation in experiments in the first series and in those in the second series so the results have been treated as a single group. In Table 1 values for uptake of O_2 at 40 min. refer to experiments from both groups, all other 40 min. values to experiments from the second group and those for 60 min. incubation to experiments from the first group.

Incubation in Ringer bicarbonate solution. A number of methods have been devised for measurement of uptake of O_2 in a bicarbonate-containing medium. Krebs's (1951) method, which makes use of the CO_2 buffers recommended by Pardee (1949), was found to be satisfactory for measurement of respiration of brain slices but was not suitable for retina. This tissue has a high rate of both respiration and aerobic glycolysis and the CO_2 produced was not absorbed sufficiently rapidly to allow accurate readings. Warburg's indirect method (1924*a*) was therefore used. A small piece of retina (15–20 mg. dry wt.) was placed in 1·5 ml. of medium in one flask and a larger piece (50–75 mg.) in 6·0 ml. in the other. Krebs–Ringer bicarbonate solution was prepared as described by Umbreit *et al.* (1945) and the appropriate amounts of glucose or pyruvate and iodoacetate were added. The solution was equilibrated with a gas mixture containing $O_2 + CO_2$ (95:5) immediately before use and the same gas mixture was passed through the flasks after they had been placed in the water bath at 37°. A period of 15 min. was allowed for gassing and temperature equilibration; pressure changes were then recorded for 25 min. During this time the rate of gas production was linear so that the total consumption of O_2 for the whole 40 min. incubation period could be calculated and compared with the lactic acid production and glucose (or pyruvate) utilization. Respiratory CO_2 was calculated by subtraction of the CO_2 equivalent of the lactic acid produced from the total CO_2 liberated.

RESULTS

Incubation in phosphate buffer. Addition of glucose greatly increased lactic acid production but not oxygen uptake during the first 40 min. incubation period (Table 1). Without glucose in the medium the rate of oxygen consumption fell off when incubation was continued for a further 20 min., but when glucose was added consumption continued at the same rate for 60 min. Although during the first 40 min. of incubation no extra oxygen was used in the presence of glucose, it is likely that some glucose was oxidized for the amount utilized was greater than that required for lactic acid production and, at glucose concentrations of 5 and 15 mM, this excess was approximately equivalent to the oxygen used. During this period, when the rate of oxygen uptake was not limited by lack of endogenous substrate, addition of glucose caused a reduction in the amount of oxygen used, but not of lactic acid produced, an effect similar to that observed by Crabtree (1929) for mouse tumours. Noell (1958) has also reported that glucose at a concentration of 20 mM causes an inhibition of 10 % in oxygen uptake of adult rabbit retina incubated in Ringer phosphate solution. Racker (1956) found a much greater effect of added glucose on the oxygen uptake of ascites-tumour cells. With this preparation endogenous respiration does not fall off during incubation for periods of 60 min., and, although at low glucose concentrations Racker found a small increase in oxygen uptake, at concentrations of 5 mM and above he found that oxygen uptake was reduced to 50–60 % of the endogenous value. In the experiments shown in Table 1, although an analysis of variance between the values for glucose concentrations of 0, 1, 5 and 15 mM showed no significant difference [F (variance ratio) $< 1{\cdot}0$], the amount of oxygen used fell continuously as the glucose concentration was increased and at a concentration of 25 mM it was significantly less than at any other concentration, being 70 % of the endogenous level.

Addition of iodoacetate to Ringer phosphate solution to give a concentration of 0·1 mM, sufficient to reduce lactic acid formation, caused no significant decrease in oxygen uptake. When the concentration was raised to 1·0 mM the decrease was significant at glucose concentrations of 0 and 15 mM ($P < 0{\cdot}01$). At a glucose concentration of 5 mM, where the values for individual experiments varied widely, the fall was not significant ($P > 0{\cdot}20$) and the low value observed at a glucose concentration of 25 mM was not further reduced by addition of iodoacetate. Glucose utilization, like lactic acid production, was

Table 1. *Effect of addition of glucose and monoiodoacetate on the oxygen consumption, lactic acid production and glucose utilization of rat retina*

Retina was incubated for 40 or 60 min. in Krebs–Ringer phosphate solution equilibrated with O_2 at 37°. Results are given as means ± S.E.M., with the number of experiments in each group shown in parentheses.

Concn. of glucose (mM)	Concn. of monoiodoacetate (mM)	O_2 uptake (μmoles/g. dry wt.)		Lactic acid production (μmoles/g. dry wt.)		Glucose utilization (μmoles/g. dry wt.)		Glucose equivalent* of O_2 uptake and lactic acid production (μmoles/g. dry wt.)	
		40 min.	60 min.	40 min.	60 min.	40 min.	60 min.	40 min.	60 min.
0	0	280 ± 17 (12)	307 ± 21 (5)	39 ± 6 (8)	32 ± 10 (5)	—	—	—	—
	0·1	284 ± 16 (10)	359 ± 24 (4)	23 ± 5 (6)	45 ± 18 (4)	—	—	—	—
	1·0	214 ± 16 (13)	224 ± 29 (5)	20 ± 4 (8)	26 ± 10 (5)	—	—	—	—
1	0	273 ± 17 (4)	416 ± 28 (4)	—	129 ± 23 (4)	—	80 ± 13 (4)	—	—
5	0	260 ± 14 (13)	351 ± 13 (6)	272 ± 43 (7)	403 ± 38 (6)	192 ± 15 (4)	221 ± 13 (6)	179	260
	0·1	208 ± 17 (7)	—	160 ± 27 (7)	—	181 ± 22 (6)	—	120	—
	1·0	219 ± 21 (12)	269 ± 27 (4)	57 ± 15 (8)	66 ± 4 (5)	109 ± 27 (5)	108 ± 9 (3)	66	78
15	0	245 ± 11 (21)	364 ± 20 (10)	245 ± 17 (11)	416 ± 40 (10)	194 ± 29 (9)	291 ± 28 (10)	176	269
	0·1	219 ± 19 (10)	379 ± 33 (3)	163 ± 23 (7)	293 ± 47 (3)	125 ± 52 (2)	156 ± 30 (3)	119	210
	1·0	198 ± 10 (11)	283 ± 11 (3)	52 ± 11 (8)	93 ± 7 (3)	119 ± 15 (4)	74 ± 19 (3)	59	94
25	0	192 ± 17 (8)	277 ± 0 (2)	362 ± 37 (6)	495 ± 88 (2)	—	—	—	—
	0·1	187 ± 22 (6)	—	197 ± 27 (6)	—	—	—	—	—
	1·0	207 ± 21 (9)	—	74 ± 17 (8)	—	—	—	—	—

* Glucose equivalent was calculated from mean values for O_2 uptake and lactic acid production.

inhibited by addition of iodoacetate, but in these conditions, where phosphate esters were likely to have been formed, the amount of glucose oxidized cannot be calculated by subtraction of the amount required for lactic acid formation from the total amount used.

Incubation in bicarbonate buffer. In this medium both oxygen consumption and lactic acid production were greater than during incubation in phosphate buffer (Table 2). When no glucose was added oxygen uptake was nearly twice as great and in the presence of glucose the value was nearly doubled again. There was no significant fall in oxygen uptake at high concentrations of glucose. When differences were large compared with the standard errors of the means statistical tests have not been applied. Endogenous lactic acid formation was not increased above the amount formed in a phosphate buffer, but when glucose was added to the medium production was much greater than in a phosphate buffer.

When no glucose was added the respiratory quotient was not significantly different from 1·0, indicating that carbohydrate was oxidized. The glycogen content of the retina before incubation was found to be 0·73 ± 0·11 mg./g. wet wt. (equivalent to 40 μmoles of glucose/g. dry wt.), somewhat lower than the value of 0·92 mg./g. found by Crane & Ball (1951*b*) and only sufficient to account for about half the endogenous oxygen uptake in bicarbonate medium. It is possible that lactic acid, produced during dissection and not removed by the preliminary soaking in cold Ringer solution, was the substrate for oxidation.

In contrast with its effect on retina incubated in Ringer phosphate solution, iodoacetate markedly inhibited oxygen uptake during incubation in bicarbonate buffer containing glucose. At an iodoacetate concentration of 0·1 mM oxygen consumption fell to about 60 % of the control value (Table 2), but an increase in concentration to 1·0 mM brought about only a small, and not significant, further decrease (for all concentrations of glucose, $P > 0·1$). Lactic acid formation was inhibited in a similar manner in both buffers, that is to a small extent at the lower and almost completely at the higher concentration. Glucose utilization was determined in only a few experiments. In the presence of iodoacetate it was reduced but, as in the phosphate experiments, the amount oxidized cannot be determined since phosphate esters may have been formed. The low respiratory quotient observed in the presence of iodoacetate could have been caused by oxidation of non-carbohydrate substrates or by an increased assimilation of carbon dioxide. The latter effect was observed by Crane & Ball (1951*b*) when iodoacetate was added to retina incubated under anaerobic conditions.

Table 2. *Effect of addition of glucose and monoiodoacetate on the oxygen consumption, lactic acid production and glucose utilization of ox retina*

Retina was incubated for 40 min. in Krebs–Ringer bicarbonate solution equilibrated with O_2+CO_2 (95:5) at 37°. Respiratory quotients were calculated from total CO_2 less CO_2 equivalent of lactic acid production. Results are given as means ± S.E.M., with the number of experiments in each group shown in parentheses.

Concn. of glucose (mM)	Concn. of monoiodo-acetate (mM)	O_2 uptake (μmoles/g. dry wt.)	Respiratory quotient	Lactic acid production (μmoles/g. dry wt.)	Glucose utilization (μmoles/g. dry wt.)	Glucose equivalent* of O_2 uptake and lactic acid production (μmoles/g. dry wt.)
0	0	494±51 (12)	0·97±0·04 (11)	40±4 (11)	—	—
	0·1	428±57 (5)	0·92±0·04 (5)	49±9 (5)	—	—
	1·0	357±81 (7)	1·01±0·07 (7)	28±5 (7)	—	—
5	0	955±70 (13)	0·98±0·02 (13)	515±36 (13)	381±43 (3)	418
	0·1	545±52 (5)	0·62±0·14 (4)	306±37 (4)	—	—
	1·0	414±54 (9)	0·89±0·13 (9)	68±17 (9)	113±12 (3)	101
15	0	872±56 (14)	0·88±0·09 (13)	424±77 (13)	199±28 (7)	357
	0·1	465±31 (7)	0·61±0·07 (6)	352±34 (6)	—	—
	1·0	379±47 (10)	0·67±0·09 (10)	125±20 (10)	—	—
25	0	885±121 (8)	0·91±0·09 (7)	522±62 (7)	—	—
	0·1	657±62 (6)	0·88±0·07 (6)	319±38 (6)	—	—
	1·0	417±187 (3)	0·73±0·21 (3)	55±20 (3)	—	—

* Glucose equivalent was calculated from mean values for O_2 uptake and lactic acid production.

Incubation with pyruvate. In Ringer phosphate solution oxygen uptake was increased above the endogenous level (in agreement with the findings of Kornblueth, Yardeni-Yaron & Wertheimer, 1953) but not to the endogenous level found in bicarbonate solution (Table 3). In bicarbonate solution addition of pyruvate brought about no rise in oxygen uptake. Addition of iodoacetate did not inhibit oxygen uptake in the presence of pyruvate, in confirmation of the results of Meyerhof & Boyland (1931), nor was lactic acid formation inhibited. Pyruvic acid utilization was apparently diminished, but in view of the small number of experiments this last result is of doubtful significance.

DISCUSSION

The high rate of oxygen consumption observed for retina incubated in bicarbonate medium containing glucose (Q_{O_2} 30·5) is similar to that found by previous workers (Warburg, 1924*b*; Laser, 1937; Craig & Beecher, 1943*a*), but the rate in phosphate buffer (Q_{O_2} 8·5) is lower than that reported by Laser and by Craig & Beecher for rat and by Auricchio (1950) for rabbit retina. Kornblueth *et al.* (1953) have suggested that bicarbonate (or carbon dioxide) is necessary for continuous synthesis of tricarboxylic acid-cycle intermediaries and they found that oxygen uptake in phosphate buffer was increased by addition of glutamate and fumarate. If the effect of carbon dioxide is only to stimulate formation of intermediary metabolites addition of other compounds forming part of the oxidative cycle should also increase oxygen uptake, but Craig & Beecher (1943*b*) did not find any increase on addition of succinate, and in our experiments pyruvate (which may be considered to be a source of cycle intermediaries) had a smaller effect than addition of bicarbonate (Table 3). Crane & Ball (1951*a*) found assimilation of carbon dioxide to be especially high in retina and suggest that malate is formed by assimilation of carbon dioxide to pyruvate with concurrent oxidation of reduced triphosphopyridine nucleotide. If this is so, the effect of bicarbonate (or carbon dioxide) on respiration may be due as much to the maintenance of a high concentration of oxidized triphosphopyridine nucleotide as to the formation of a necessary intermediary. Bicarbonate also increases lactic acid formation although the ratio of lactic acid produced to oxygen consumed is less in bicarbonate than in phosphate buffer (Table 4). If assimilation of carbon dioxide results in an increase in metabolism of pyruvate then reactions which produce pyruvate may also be stimulated and thus more pyruvate be available for reduction to lactate. It is possible that such a shift in reaction velocities could result in an increase in lactate formation although, at the same time, the ratio of lactate formed to oxygen utilized might fall.

Addition of iodoacetate had little effect on oxygen uptake during incubation in phosphate buffer. A similar lack of effect in other tissues has been interpreted by some workers (e.g. Barker *et al.* 1939) as evidence for oxidation by a non-glycolytic pathway, but the lack of inhibition may well be a

Table 3. *Effect of addition of pyruvate and monoiodoacetate on the oxygen consumption, lactic acid production and pyruvate utilization of ox retina*

Retina was incubated for 40 or 60 min. in Krebs–Ringer phosphate or bicarbonate solution at 37°. Results are given as means ± S.E.M., with the numbers of experiments in each group shown in parentheses.

Concn. of pyruvate (mM)	Concn. of monoiodoacetate (mM)	O_2 uptake (μmoles/g. dry wt.)		Lactic acid production (μmoles/g. dry wt.)		Pyruvate utilization (μmoles/g. dry wt.)		Pyruvate equivalent* of O_2 uptake and lactic acid production (μmoles/g. dry wt.)	
		40 min.	60 min.	40 min.	60 min.	40 min.	60 min.	40 min.	60 min.
				(*a*) Krebs–Ringer phosphate					
0	0	280 ± 17 (12)	307 ± 21 (5)	39 ± 6 (8)	32 ± 10 (5)	—	—	—	—
5	0	387 ± 42 (7)	557 ± 25 (7)	—	128 ± 13 (7)	—	304 ± 25 (7)	—	351
	0·1	351 ± 9 (4)	506 ± 19 (4)	—	172 ± 41 (4)	—	327 ± 23 (7)	—	375
	1·0	305 ± 11 (2)	415 ± 9 (2)	—	124 ± 23 (2)	—	233 ± 7 (2)	—	290
				(*b*) Krebs–Ringer bicarbonate					
0	0	494 ± 51 (12)	—	40 ± 4 (8)	—	—	—	—	—
5	0	461 ± 62 (8)	—	97 ± 16 (8)	—	347 ± 17 (2)	—	282	—
	1·0	524 ± 26 (4)	—	167 ± 19 (4)	—	251 ± 21 (4)	—	376	—

* Pyruvate equivalent was calculated from mean values for O_2 uptake and lactic acid production.

Table 4. *Comparison of lactic acid produced and oxygen consumed during incubation of retina in Ringer phosphate and bicarbonate solutions containing glucose*

See Tables 1 and 2.

Concn. of glucose (mM)	Lactic acid produced (μmoles/g. dry wt.)	Oxygen used (μmoles/g. dry wt.)	Ratio (lactic acid produced : oxygen consumed)
Incubation in Ringer phosphate solution for 40 min.			
5	272	260	1:0·96
15	245	245	1:1·00
25	362	192	1:0·53
Incubation in Ringer phosphate solution for 60 min.			
5	403	351	1:0·87
15	416	364	1:0·88
25	495	277	1:0·56
Incubation in Ringer bicarbonate solution for 40 min.			
5	515	955	1:1·85
15	424	872	1:2·06
25	522	885	1:1·68

reflexion of a submaximal rate of oxidation, an explanation suggested by Heald (1953) to account for his observation that iodoacetate inhibited oxygen uptake of stimulated but not of resting brain tissue. Oxygen uptake of retina incubated in bicarbonate buffer was strongly inhibited by addition of iodoacetate but only when glucose was also present. This suggests that the effect was due to inhibition of glucose utilization rather than of some step in the oxidation process. Hexokinase in retina, as in other tissues, is partially inhibited by low concentrations of iodoacetate (D. S. Hoare, personal communication), so that in conditions where glucose utilization is high iodoacetate would be expected to reduce the amount used, whereas when utilization is low, as during incubation in phosphate buffer, sufficient may still be phosphorylated to allow oxidation, but not glycolysis, to continue uninhibited. The inhibition of lactic acid production would in these conditions be due to inhibition of triose phosphate dehydrogenase activity. The difference in effect of iodoacetate on oxygen uptake of retina in phosphate and bicarbonate buffers cannot therefore be used as evidence for the occurrence of different oxidative pathways in the two media, but this difference in effect in different metabolic conditions may perhaps explain the conflicting reports of previous workers who have studied the effect of iodoacetate on oxidative reactions.

SUMMARY

1. The rate of oxygen consumption of ox retina incubated in Ringer bicarbonate solution is increased by the addition of glucose but not by that

of pyruvate. When retina is incubated in Ringer phosphate solution the reverse is the case, for in the presence of glucose the rate of oxygen consumption is slightly depressed, although the initial rate is maintained for a longer period; addition of pyruvate causes a small rise in oxygen consumption.

2. Addition of monoiodoacetate at a concentration of mM causes only a small reduction in the amount of oxygen used during incubation in Ringer phosphate solution, although lactic acid production is strongly inhibited.

3. During incubation in Ringer bicarbonate solution containing glucose oxygen consumption is inhibited by 40% in a concentration of 0·1 mM-iodoacetate; an increase in concentration of iodoacetate to 1·0 mM does not bring about a significant further reduction in oxygen uptake.

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The Fatty Acids of Human Milk from Mothers on Diets Taken *ad libitum**

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The unique position of milk as a primary food and as a readily sampled product of active lipid metabolism emphasizes the desirability of obtaining detailed information on human-milk fatty acids. Fatty acids as glycerides comprise the bulk of milk lipids, less than 2% of which are cholesterol and phospholipid (Macy, Kelly & Sloan, 1953). The non-esterified fatty acids of milk, constituting about 2% of total milk lipids, have received slight attention. If analogous to those in serum (Dole, 1956; Gordon & Cherkes, 1956) they might have considerable physiological importance.

The major fatty acids of human milk lipids have been studied in a number of Laboratories (Bosworth, 1934; Hilditch & Meara, 1944; Baldwin & Longenecker, 1944; Brown & Orians, 1946). Analyses made by fractional distillation of the methyl esters, followed by crystallization and in some instances by spectroscopy, required sizeable quantities of starting materials; pooled milk specimens were used in all cases. Although studies on individual milks have not been feasible in such detail, Söderhjelm (1953) has defined the di- to hexa-ene contents of individual samples by ultraviolet spectrophotometry after isomerization with alkali. With gas–liquid chromatography of the methyl esters of fatty acids, introduced by James & Martin (1956) and improved by Lovelock & James (1959), precise all-inclusive analyses now can be made on multi-component mixtures totalling 50 μg. or less. Thus it becomes timely to refine the study of lactation by examining individual milks, in order to avoid any imprecisions due to pooling of samples.

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The present paper lays a base for further study of physiological variations in human lactation by defining the fatty acid composition of the milk lipids of eleven women on diets taken *ad libitum*. Analyses were made by the combined approach of spectrophometry and gas–liquid chromatography.

METHODS

Clinical procedure

Milk sampling. Specimens of milk were obtained from six normal women in hospital with their breast-fed newborn infants. These stays in hospital were arranged solely for carrying out biochemical studies on lactation, and the patients were confined to a rooming-in unit in a metabolic ward. Breast feedings were made every 3–4 hr., alternating the breast initially nursed. To make 24 hr. collections, nursing was interrupted and the breasts were emptied at regular nursing times with an electric pump, followed by manual expression as described by Davies (1945). Of the milk collected at each breast 10% was taken for analysis and the remainder fed by bottle to the infant. The individual samples were combined in a pool representing the 24 hr. production of each breast. All milk samples were refrigerated immediately after collection and the final pools were stored at −15°.

In addition, random specimens of milk were obtained from five women living at home. Milk was collected by manual expression from one or both breasts at the end of one or more feedings. The infants of these mothers all received varying amounts of supplementary feedings with milk substitutes or prepared infant foods. In these cases determinations of 24 hr. milk volumes were not made. All samples were refrigerated immediately upon collection and upon delivery to the Laboratory were stored at −15°.

Maternal diets. The patients in hospital were offered a diet of solid foods containing 2200–3000 kcal. with three servings of protein a day. Two of these servings were meat and one was egg, but milk or cheese was provided with each meal bringing the protein intake to 15% of total calories. Fat constituted about 40% of total calories, about half from milk and butter, one-third from meat and eggs and the remainder from vegetables. Precise food consumptions were not determined. The diets of mothers living outside the hospital were not controlled; however, they were not limited by economic factors.

Chemical methods

Extraction of lipids. The lipids in a sample (1 or 2 ml.) of milk were extracted with 25 vol. of 95% ethanol–ethyl ether (3:1, v/v). Solvent was removed from the filtered extract by low-temperature vacuum-distillation (Craig, Gregory & Hausmann, 1950). Extraction of the residue with light petroleum (b.p. 60–70°) yielded an extract free of non-lipid contaminants. The content of total lipids was determined microgravimetrically as described by Craig, Hausmann, Ahrens & Harfenist (1951). After saponification by refluxing with 5% KOH in absolute methanol for 2 hr., the mixture was acidified and the fatty acids were recovered. Their amount was determined microgravimetrically by the procedure of Ahrens & Craig (1952), in which only the long-chain acids remain to be weighed. All solvents were glass-distilled.

Recovery of fatty acids averaged 92% of the total lipid (theoretical recovery, 95·5% if all lipid were triolein), with losses to be expected during saponification and extraction of the acidified medium. The minimal disagreement between actual and theoretical recoveries indicated that short-chain water-soluble fatty acids (which would have been disregarded by our analytical method) were present only in very small amounts if at all.

The effectiveness of this extraction procedure was evaluated by comparison with three other methods: ethanol–ether extraction of Somogyi's (1930) zinc hydroxide coprecipitate of protein and lipid, chloroform–methanol extraction (Folch, Ascoli, Lees & Meath, 1951) followed by light petroleum rectification, and counter-current distribution (five transfers in a heptane–ether–ethanol–water system) as described by Blankenhorn & Ahrens (1955). Total lipids calculated from gravimetric results were essentially the same by all four methods. However, the small amount of phospholipid present in milk was most effectively extracted by the chloroform–methanol method (84 mg./100 ml. of milk) and less well by direct ethanol–ether extraction with rectification (42 mg.) and by extraction of the zinc-precipitated lipoproteins (12 mg.); the countercurrent-distribution procedure had been designed to avoid this material completely. In view of the small amounts of phospholipid we found in milk, also shown by Macy *et al.* (1953) and by Morrison (1952), our choice of the direct ethanol–ether method was based on merits of technical simplicity. Agreement of the results for total lipid obtained by the four methods indicated that all extracts were free of non-lipid material.

Analysis of fatty acid. The mixed fatty acids were analysed for their content of polyene classes by spectrophotometry and for concentrations of individual acids by gas–liquid chromatography. Alkali isomerization and ultraviolet spectrophotometry were performed on a microscale in duplicate according to Holman & Hayes (1958) and were corrected for preformed conjugation. For gas–liquid chromatography the acids were methylated by refluxing with 5% (v/v) conc. HCl–absolute methanol for 2 hr., but in some experiments the milk glycerides were methylated directly by inter-esterification with HCl–'superdry' methanol as described by Stoffel, Chu & Ahrens (1959). The methyl esters of C_{6-18} acids were chromatographed at 197° on a non-polar stationary phase, Apiezon M, with nitrogen as the mobile phase, with the gas-density balance detector of Martin & James (1956). Methyl esters of C_{19-22} acids were analysed by chromatography at 197° on Orr & Callen's (1959) polar stationary phase, Reoplex 400. The ionization chamber of Lovelock & James (1959) was used as a sensing device. The column efficiency with Apiezon was 3500–4000 theoretical plates, with Reoplex 2100 plates, calculated for methyl stearate according to Desty (1957). Separation factors of methyl oleate and stearate were 1·18 on Apiezon and 1·12 on Reoplex. The complementary data from these two chromatographic analyses were combined by using ratios of single components common to both. Major acids were identified by comparison with the retention volumes of pure standards, all volumes being measured from the air peak. A number of pure polyethenoid acids were identified on the basis of structural studies and chromatographic characteristics carried out in this Laboratory by Stoffel, Insull & Ahrens (1958).

RESULTS

The sources of the milk samples are described in Table 1. The six mothers in the hospital (mean age of 22 years) had been lactating for approximately 12 days. There was considerable variation from patient to patient in volumes of milk secreted in 24 hr. and in concentrations of milk fat; moreover, the simultaneous yields of the two breasts were not similar. However, the values of milk and milk-fat production are all within the normal ranges recorded by Macy *et al.* (1953) and by Morrison (1952). The five out-patients (mean age of 27 years) had been lactating for 35–180 days. The fat concentration in their milk samples was lower than that in the milk of the hospitalized patients.

The proportions of polyene acids in the milk fatty acids, determined by alkali-isomerization and spectrophotometry, are shown in Table 2. All polyenes from dienes to hexaenes were present. Analyses for the two groups of patients were very similar in concentrations of total polyenes: 9·9% of total acids in the hospitalized patients, 10·3% in the out-patients. The in-patients had a lower mean proportion of dienes and higher mean proportions of trienes, tetraenes, pentaenes and hexaenes, but the differences were not statistically significant. In each patient milks collected simultaneously from the two breasts showed almost identical polyene analyses.

Gas–liquid chromatography of the fatty acid methyl esters revealed the presence of at least thirty-nine different acids (Table 3). Many of these could not be identified on the basis of retention

Table 1. *Description of individual human milk samples collected from eleven normal mothers on diets taken* ad libitum

Clinical data on mothers					Milk samples				
Patient number, initials and age (years)			No. of pregnancy	Study environment	Period post-partum (days)	Volume (ml./24 hr.)		Fat content (g./100 ml. of milk)	
						Right breast	Left breast	Right breast	Left breast
1	R.Q.	20	1	Metabolic ward	21	67	—	4·68	—
2*	H.L.	21	1		10	534	413	5·09	5·47
3*	N.W.	21	1		15	228	338	2·04	2·16
4	M.T.	23	1		9	239	185	2·27	1·98
5*	C.M.	23	1		11	243	287	1·37	1·79
6	E.M.	23	3		10	464	335	3·38	3·07
7	P.E.	20	2	Home	35	—		0·81	
8	J.H.	27	1		130	—		0·63	
9	L.P.	27	3		60	—		0·96	
10	E.J.	29	1		40	—		2·14	
11	P.I.	30	1		180	—		0·66	

* Negro patient.

Table 2. *Polyene acids as percentages of total fatty acids*

Separate analyses of milks of right and left breasts were averaged in patients 2–6.

Patient number	Dienes	Trienes	Tetraenes	Pentaenes	Hexaenes	Total polyenes	Trihexaenes
				In-patients			
1	7·91	1·32	0·72	0·28	0·32	10·55	2·64
2	8·39	1·11	0·68	0·26	0·35	10·79	2·40
3	5·84	1·20	0·73	0·28	0·20	8·25	2·41
4	7·97	1·28	0·89	0·36	0·45	10·95	2·98
5	6·57	1·15	0·73	0·28	0·33	9·06	2·49
6	7·69	1·02	0·69	0·30	0·34	10·04	2·35
Mean	7·40	1·18	0·74	0·29	0·33	9·94	2·55
S.D.	2·19	0·25	0·17	0·08	0·18	2·40	0·53
				Out-patients			
7	7·15	0·99	0·60	0·23	0·20	9·17	2·02
8	6·45	1·10	0·42	0·23	0·05	8·25	1·80
9	11·00	1·22	0·45	0·21	0·08	12·96	1·96
10	7·70	1·00	0·48	0·19	0·20	9·57	1·87
11	9·99	0·76	0·40	0·21	0·31	11·67	1·68
Mean	8·46	1·01	0·47	0·21	0·17	10·32	1·87
S.D.	3·89	0·34	0·16	0·03	0·21	3·87	0·27

Table 3. *Fatty acid patterns in milk lipids of eleven mothers on diets taken* ad libitum

Values are expressed as percentages of total fatty acid methyl esters. Current assignment of structure is given under 'Shorthand designation'. Retention volumes on two stationary phases were determined as described by Stoffel, Insull & Ahrens (1958).

Fatty acid																			
			Retention volumes relative to methyl stearate														Means		
					In-patients						Out-patients								
No.	Familiar name	Shorthand designation	Apiezon M	Reoplex 400	1	2	3	4	5	6	7	8	9	10	11	In-patients (6)	Out-patients (5)	Entire group (11)
1	—	<10: 0	—	—	0·50	0·31	0·22	0·11	0·20	0·15	0·10	0·08	0·18	0·21	0·86	0·25	0·29	0·26
2	Capric (decanoic)	10: 0	0·029	0·10	2·80	1·66	2·39	1·05	2·17	0·81	1·42	0·92	1·57	1·98	1·13	1·81	1·40	1·63
3	Undecanoic	11: 0	0·047	0·13	—	—	—	—	—	—	—	0·01	—	—	—	—	0–0·01	0–0·01
4	—	12: hbr?	0·054	—	—	—	—	—	—	—	—	0·01	—	—	—	—	0–0·01	0–0·01
5	Lauric	12: 0	0·075	0·16	8·46	8·36	9·99	3·82	6·34	5·02	4·89	4·86	7·07	10·75	5·63	7·00	6·64	6·84
6	—	12: br?	0·067	—	—	—	—	—	—	—	—	0·04	—	—	—	—	0–0·04	0–0·04
7	Tridecanoic	13: 0	0·112	0·22	—	—	0·06	—	—	—	—	0·03	—	0·09	—	0–0·06	0–0·06	0–0·06
8	Myristic	14: 0	0·174	0·31	9·31	10·13	12·11	5·99	8·19	6·84	5·94	8·04	10·14	9·85	7·21	8·76	8·24	8·52
9	Myristoleic	14: 1	0·159	0·36	0·30	—	0·17	0·20	0·36	0·35	0·23	0·26	0·26	0·18	0·10	0–0·27	0·21	0·24
10	—	14: ?	0·147	—	tr.	—	—	—	0·11	—	0·05	0·09	—	—	0·08	0–0·11	0–0·07	0–0·08
11	Pentadecanoic	15: 0	0·270	0·41	0·49	0·63	0·43	0·50	0·59	0·67	0·58	0·53	0·58	0·43	0·44	0·55	0·52	0·53
12	—	15: br?	0·233	—	0·37	0·30	0·52	0·53	0·48	0·47	0·30	0·27	0·33	0·28	0·39	0·45	0·31	0·39
13	Palmitic	16: 0	0·419	0·56	21·48	19·12	20·62	21·79	19·93	23·30	21·11	18·92	24·58	17·64	21·17	21·04	20·68	20·88
14	Palmitoleic	16: 1	0·366	0·63	1·94	1·69	1·74	3·00	1·81	3·17	3·41	3·63	2·45	1·83	2·07	2·23	2·68	2·43
15	Heptadecanoic	17: 0	0·649	0·74	0·88	0·36	0·62	0·31	0·48	0·53	0·33	0·39	0·35	0·14	0·70	0·53	0·38	0·46
16	—	17: br?	0·555	—	0·54	0·69	0·47	0·53	1·12	0·99	0·57	0·87	0·76	0·57	0·87	0·72	0·73	0·73
17	Stearic	18: 0	1·00	1·00	6·42	7·50	6·98	6·60	7·88	8·04	6·48	7·74	7·67	6·43	8·95	7·24	7·45	7·34
18	*iso*Oleic	18: 1 iso	0·896	<1·12	6·84	5·42	9·05	7·83	6·53	5·02	8·10	4·09	3·84	8·64	6·10	6·78	6·15	6·50
19	Oleic	18: 1	0·860	1·12	28·36	25·85	25·93	33·26	30·25	29·63	32·41	32·49	24·14	29·37	31·05	28·88	29·89	29·34
20	Linoleic	18: 2	0·801	1·33	5·63	7·60	4·27	7·00	6·61	6·96	7·71	8·83	9·76	6·43	7·24	6·35	7·99	7·09
21	—	*un*	—	1·48–1·50	0·82	0·84	0·29	0·06	0·56	0·69	0·71	0·81	0·99	0·81	0·78	0·54	0·82	0·67
22	Linolenic	18: 3	0·801	1·67–1·69	0·55	1·07	0·54	0·98	0·99	0·93	0·90	0·98	0·67	0·77	1·02	0·84	0·87	0·85
23	—	20: 0	2·38	1·76–1·78	0·55	1·60	0·95	1·17	1·46	1·60	1·30	1·68	1·05	1·99	1·04	1·22	1·41	1·31
24	—	20: 1	1·99	1·97–1·99	0·75	1·55	0·59	1·06	0·80	1·10	0·84	0·99	0·64	0·67	0·84	0·98	0·80	0·89
25	—	*un*	—	2·10–2·14	0·59	0·35	0·24	0·33	0·20	0·33	0·25	0·25	0·18	0·27	0·20	0·35	0·23	0·30
26	—	20: 2?	—	2·24–2·26	0·73	0·23	0·16	0·36	0·24	0·25	0·15	0·20	0·12	0·21	0·15	0·33	0·17	0·25
27	—	21: 0	—	2·38–2·40	0·46	0·93	0·28	0·58	0·38	0·67	0·36	0·49	0·46	0·21	0·50	0·55	0·40	0·48
28	—	20: 3	1·64	2·68–2·70	0·31	0·81	0·19	0·63	0·36	0·49	0·26	0·34	0·59	0·34	0·20	0·47	0·35	0·41
29	Arachidonic	20: 4	1·46	2·95–2·98	0·21	1·17	0·34	0·89	0·72	0·69	0·53	0·43	0·52	0·39	0·38	0·67	0·45	0·57
30	—	*un*	—	3·18–3·25	0·05	0·20	0·09	0·15	0·10	0·14	0·12	0·15	0·09	0·08	0·05	0·12	0·10	0·11
31	—	22: 1?	—	3·40–3·41	—	tr.	—	—	0·07	—	tr.	—	0·09	0·06	—	0–0·07	0–0·08	0–0·07
32	—	*un*	—	3·56–3·60	0·06	0·08	0·04	0·16	0·10	0·11	0·08	0·79	tr.	0·08	0·09	0·09	0·26	0·16
33	—	20: 5	1·46	3·69–3·78	—	0·05	0·04	0·12	0·10	0·08	0·04	0·07	0·23	0·02	—	0–0·08	0–0·09	0–0·08
34	—	*un*	—	4·25–4·33	0·02	1·20	0·05	—	tr.	0·06	0·08	0·10	0·05	—	0·05	0–0·33	0–0·07	0–0·20
35	—	*un*	—	5·32–5·39	0·09	0·24	0·09	0·16	0·11	0·09	0·09	0·19	0·10	0·06	0·09	0·13	0·11	0·12
36	—	*un*	—	5·74–5·90	0·10	0·33	0·16	0·10	0·09	0·24	0·17	—	0·23	0·04	0·13	0·17	0–0·14	0–0·16
37	—	*un*	—	6·35–6·42	—	tr.	—	tr.	—	0·09	—	—	—	—	0·17	0–0·09	0–0·17	0–0·13
38	—	22: 5	3·30	6·75–6·85	0·06	0·15	0·13	0·19	0·14	0·09	0·10	0·17	0·18	0·06	0·13	0·13	0·13	0·13
39	—	22: 6	3·00	7·51–7·60	0·17	0·62	0·21	0·50	0·45	0·34	0·30	0·29	0·12	0·13	0·21	0·38	0·21	0·30

volumes established to date, and are shown in Table 3 in terms of retention volumes on Reoplex relative to methyl stearate. Since the normal saturated C_{20} acid has a retention volume of $C_{18} \times 1{\cdot}78$, whereas $n\text{-}C_{22} = C_{18} \times 3{\cdot}17$ and $n\text{-}C_{24} = C_{18} \times 5{\cdot}65$, it is evident that most of the unidentified acids are $C_{18\text{-}22}$ unsaturated acids. Eight acids constituted 88·4 % of the total; oleic 29, palmitic 21, myristic 9, linoleic 7, stearic 7, lauric 7, *iso*oleic 7, and palmitoleic acid 2 % of the total. Degradation studies carried out according to James & Webb (1957) showed that a significant part of the *iso*oleic acid is octadeca-11-enoic acid. This acid appeared as a shoulder on the descending limb of the oleate curve on Apiezon, but was poorly resolved from oleic acid on Reoplex. Only 0·5 % of the total acids were C_{10} or shorter. About 2 % of the total acids were odd-numbered, and most of these were C_{15} and C_{17} acids.

About 5 % of total fatty acids were $C_{19\text{-}22}$; they numbered 17 acids. A number of C_{20} and C_{22} unsaturated acids were recognized which had previously been identified in menhaden body oil by Stoffel *et al.* (1958). These are shown in Table 3 by specific numerical designations. At least eight other unsaturated acids, marked *un* in Table 3, have not been characterized by chain length or double-bond structure.

DISCUSSION

Analysis of the polyethenoid fatty acids of milk by spectrophotometry and gas–liquid chromatography offers an unique opportunity for rigorous comparison of these two techniques. Results on a variety of human milks obtained under experimental conditions (W. Insull & E. H. Ahrens, unpublished work) are presented in Table 4. Since linoleate and linolenate overlap when gas–liquid chromatography is carried out on Apiezon M, the sum of these two esters was compared by the two methods of analysis; agreement in results is seen to be excellent. By contrast, when chromatography is carried out on Reoplex 400, the linoleate–linolenate overlap is easily resolved. In addition, the entire range of polyenes can be evaluated by the two methods, the results in Tables 2 and 3 being used. This comparison (Table 5) is made with the full realization that many of the $C_{19\text{-}22}$ polyenes remain unidentified and that the extinction coefficients used for calculation of these polyenes have not been determined. Nevertheless the two methods of analysis give results on the proportions of trienes, tetraenes, pentaenes and hexaenes which are reassuringly similar in order of magnitude.

Our analyses of individual milk specimens by gas–liquid chromatography agree with previous studies of pooled milk samples in the proportions of major components (Table 6). Furthermore, analyses on the milks of the two breasts collected simultaneously were nearly identical in every instance. The data assembled from the literature indicate that eight acids characteristically constitute about 90 % of the total acids in human milk. The total of these eight acids was only 89 % in the present study, the lowest yet reported, perhaps because our analyses demonstrated a number of acids previously overlooked. $C_{6\text{-}9}$ acids were present only in traces, and there was indirect evidence that acids shorter than C_6, if present,

Table 4. *Comparison between gas–liquid chromatographic and spectrophotometric estimations of dienes and trienes in human milk*

Results are expressed as percentages of total acids. All differences (last column) were statistically insignificant.

Source of fat in maternal diet	Number of analyses	Spectrophotometric			Chromatographic (Linoleic + linolenic)	Difference between methods
		Dienes	Trienes	Di- + tri-enes		
Mixed diet	11	8·41	1·05	9·46	8·96	−0·50
Maize oil	12	41·62	1·15	42·77	42·88	+0·11
Linseed oil	6	8·09	36·58	44·67	42·95	−1·72

Table 5. *Comparison between gas–liquid chromatographic and spectrophotometric estimations of all polyenes in human milks from eleven mothers on diets taken* ad libitum

Results are expressed as percentages of total acids.

Method	Dienes	Trienes	Tetraenes	Pentaenes	Hexaenes
Spectrophotometric (Table 2)	7·88	1·10	0·62	0·25	0·26
Gas–liquid chromatography (Table 3)*	7·34	1·26	0·57	0·21	0·30

* Totals from Table 3 are: dienes (acids 20 + 26); trienes (acids 22 + 28); tetraenes (acid 29); pentaenes (acids 33 + 38); hexaenes (acid 39).

occurred only in very small proportions. Monoenoic C_{10-18} acids were found regularly. Odd-numbered acids C_{9-17} were always present, as noted by James, Webb, Stapleton & McDonald (1958). Long-chain branched acids, isolated and positively identified by Shorland (1955) in butter and mutton fat, were recognized by James and co-workers (James *et al.* 1958) in their gas–liquid chromatographic analyses of goat milk and human sebum (James & Wheatley, 1956); our results suggest their presence in human milk in small amounts. We have noted a wide array of C_{19-22} unsaturated acids and the regular occurrence of one or more oleic acid isomers.

The lactation of eleven in- and out-patients was fully established, as judged by Morrison's evaluation of daily milk-production figures. Since samples from the in-patients were obtained after 10 days of lactation whereas the out-patients had been lactating 35–180 days, some interesting comparisons can be made between the two groups. Despite wide differences in fat content of the milk samples of the two groups, the fatty acid compositions differed only to a minor degree. The question can be raised whether the random samples obtained from the out-patients group can properly be compared with the truly representative samples of 24 hr. output of the in-patients. A partial answer to this query is found in our preliminary comparisons of fatty acid patterns of milks obtained at the onset and at the end of a nursing period: no differences were seen. Nor have we been able to demonstrate any diurnal variation in milk-fatty acid patterns of mothers fed with experimental diets containing 40% of calories as fat, although some variation was seen when mothers ingested fat-free diets.

The present analyses of the proportions of major and minor components in human milk of mothers fed *ad libitum* form a basis for comparison with milks produced under experimental conditions. Studies by Insull, Hirsch, James & Ahrens (1959) have shown that wide variations in human-milk-fat composition can be produced by alterations of caloric intakes as well as by exchange of dietary fats. Under rigidly controlled experimental conditions it was demonstrated that dietary fatty acids are rapidly transferred into the milk lipids. Thus within 2 or 3 days the character of the breast-milk fat can be radically changed to mimic the dietary fat. Moreover, when too few calories are fed to maintain the mother's body weight, adipose tissue fatty acids are rapidly transferred into the milk lipids. However, when excess of calories of a fat-free diet are fed, synthesis of fat from carbohydrate occurs in the breast, and milk of a very distinctive type is produced, poor in all unsaturated acids and rich in C_{12}, C_{14} and C_{16} saturated acids.

With these facts in mind it may seem remarkable that the milk fatty acids of mothers on diets taken *ad libitum* are so similar, whether in the early or late post-partum period. In part, the resemblances between the data presented by four Laboratories (Table 6) might be due to qualitative similarities in the dietary fats of the mothers who furnished the milk for these studies. Excluding the unusual circumstance of a nursing mother gaining weight on a very low fat diet, it is reasonable to assume that under ordinary conditions the major determinant of milk-fatty acid composition is the nature and quantity of the maternal dietary-fat intake. However, from one meal to the next there must be marked qualitative differences in maternal dietary fats. Nevertheless, the full effect of these alterations is not manifested in the breast milk within hours, but only in days. Thus the irregularities due to varying intakes are evened out by the time the dietary fatty acids are transported into the milk. It seems necessary to postulate that the dietary fatty acids are first transported into some intermediate pool of considerable size and later into the

Table 6. *Major fatty acids in human milk*

Results are expressed as percentages (by wt.) of total fatty acid methyl esters.

	Pooled milk			
Acid	Baldwin & Longenecker (1944) (mature milk)	Hilditch & Meara (1944) (late milk)	Brown & Orians (1946) (sample I)	Present results (averages of 11 individual samples)
Lauric	5·5	7·0	5·4	7·0
Myristic	8·5	13·9	6·7	8·8
Palmitic	23·2	24·1	21·0	21·0
Palmitoleic	3·0	2·8	2·7	2·2
Stearic	6·9	9·6	6·8	7·2
Oleic	36·5	30·2	39·1	35·8*
Linoleic	7·8	5·5	10·3	6·4
Total	91·4	93·1	92·0	88·4

* Total of acids numbered 18 and 19 in Table 3.

milk. Whether this pool is the mammary gland itself or some other depot fat cannot be answered at present. However, in such a pool the qualitative differences in the fatty acids eaten at one or another time would be averaged. Thus the milk lipids, formed predominantly by transference from this pool, must represent the last stage of a process which is not unlike that of a 'moving average' in statistics. The advantage of this seemingly homeostatic mechanism to the child is obvious.

SUMMARY

1. The fatty acid compositions of human milk obtained on individual samples from eleven mothers on diets taken *ad libitum* were determined by gas–liquid chromatography and by ultraviolet spectrophotometry after isomerization of polyenoic acids with alkali.

2. The patterns of acids from the two breasts were nearly identical. A major proportion (88·4%, calculated as the methyl esters) was made up of eight components: oleic 29%, palmitic 21%, myristic 9%, linoleic 7%, stearic 7%, lauric 7%, *iso*oleic 7%, and palmitoleic 2% of the total. However, at least 30 other acids were seen in minor amounts, odd-numbered and branched-chain acids as well as a wide variety of C_{19-29} unsaturated acids. The two complementary methods of analysis produced results in reasonable agreement.

3. The fatty acid composition of milks of mothers in the early and late post-partum periods were very similar, although the fat concentrations of the samples differed greatly.

4. It is suggested that the similarities in milk-fatty acid patterns of the in- and out-patients were due in part to similarities in the quality of dietary fat ingested by the two groups. However, in addition, the existence of a pool intermediate between dietary fat and milk fat is postulated, which acts to buffer radical changes in milk-fatty acid composition under ordinary circumstances.

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Amino Acid Incorporation into Cell-Free Preparations of Normal and Neoplastic Lymphatic Tissues of the Rat

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Zamecnik & Keller (1954) showed that it was possible to obtain an energy-dependent incorporation of amino acids into protein when microsomes isolated from rat liver were incubated with soluble cytoplasm and radioactive amino acids. These experiments encouraged the hope that an understanding of the way in which proteins are synthesized by the cell might emerge from studies involving the incubation of subcellular particles. Ultimately it will, of course, be necessary to devise the means whereby the synthesis of specific proteins can be followed in such experiments, but before this is possible the requirements for the incorporation of amino acids into the protein of particulate fractions of many different tissues, each capable of synthesizing a different specific protein, must be elucidated. Some progress in this direction has been reported (e.g. Weiss, Acs & Lipmann, 1958).

It was decided to investigate the lymphatic tissues, because of their known ability to synthesize γ-globulin and antibody. In 1957, Wagle, Mehta & Johnson reported that nuclei-free suspensions isolated from rat spleen retained incorporating activity (see also Wagle, Mehta & Johnson, 1958). In preliminary experiments, preparations from spleen, lung and bone marrow therefore were investigated, but for various reasons most of the work to be reported in the present paper has involved the spleen. The incorporation of amino acids into preparations from transplanted rat lymphosarcoma has also been studied, for not only was it of interest to determine whether the tumour tissue differed from other lymphatic tissues in this respect, but also it seemed possible that a tumour tissue might offer interesting advantages as experimental material.

When a rat is injected with radioactive amino acids the most rapid incorporation of radioactivity in the liver is into the microsomal fraction of the cell (Keller, Zamecnik & Loftfield, 1954). Furthermore, when the subcellular fractions are isolated and incubated with a radioactive amino acid the energy-dependent incorporation of amino acid into the microsome fraction is greater than that occurring in the other cell fractions. On the other hand, if the extent of incorporation of radioactive amino acids into the subcellular fractions of the spleen is determined 20 min. after the injection of amino acid the mitochondrial fraction is the most active (Campbell, Greengard & Rendi, 1958). This suggested that this fraction of the spleen might also be the most active fraction under conditions *in vitro*. However, it will be shown that, as with the liver, the microsomal and not the mitochondrial fraction of the spleen is the more active.

In an attempt to determine whether there is any characteristic difference in the incorporation of amino acids into the microsomal fraction of the liver and spleen the extent of incorporation of various amino acids into this fraction of the two tissues has been compared. In fact, such differences were found and these have been used to study the role of the cell-sap fraction in determining the specificity of the incorporation of amino acids into the microsomes.

A preliminary report of some aspects of this work has already appeared (Rendi & Campbell, 1958).

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MATERIALS AND METHODS

Chemicals. Adenosine triphosphate (dipotassium salt; ATP) and guanosine triphosphate acid (sodium salt; GTP) were obtained from Sigma Chemical Co., St Louis, Mo., U.S.A. and neutralized with KOH. The barium silver salt of phosphoenolpyruvic acid (PEP) was prepared by the method of Ohlmeyer (1951), and the dibarium salt of fructose diphosphoric acid (FDP) was obtained from C. H. Böhringer und Söhne G.m.b.H., Mannheim, Germany. A solution of the free acids was prepared shortly before use and was adjusted to pH 7·8. Glutathione was obtained from The Distillers Co. (Biochemicals) Ltd., Liverpool. PEP-kinase, obtained either from C. H. Böhringer und Söhne, or prepared from rabbit muscle as described by Bücher & Pfleiderer (1955), was dialysed against twice-distilled water and diluted so that the protein concentration was 1 mg./ml.

Radioactive amino acids. Uniformly labelled [^{14}C]glycine, L-[^{14}C]alanine, L-[^{14}C]leucine, L-[^{14}C]lysine and L-[^{14}C]-glutamic acid were obtained from The Radiochemical Centre, Amersham, Bucks.

Tissues. Spleen, liver and bone marrow were obtained from Wistar albino rats fed on stock diet. For spleen and liver, rats of 150 g. were used; for bone marrow rats of 250–350 g. were used. The bones of the limbs of the rats

were prepared as free from muscle as possible, washed in cold saline and split with a forceps. The bone marrow was suspended with a glass rod in saline and washed twice. Rats bearing transplantable Murphy lymphosarcoma were obtained from the Imperial Cancer Research Fund Laboratories at Mill Hill, London.

Tissue preparations. (1) Cell-free suspensions. Spleen, liver and lymphosarcoma were homogenized with 2·5 vol. of a medium containing 0·01 M-$MgCl_2$, 0·03 M-$KHCO_3$, 0·025M-KCl, 0·02M-potassium phosphate buffer, pH 7·8, and 0·35 M-sucrose, in an all-glass Potter homogenizer, and packed bone-marrow cells were disrupted by freezing and thawing and then suspended in 2·5 vol. of the medium with the aid of an homogenizer. The suspensions were centrifuged at 2° for 10 min. at either 5000 *g* or 12 000 *g*.

(2) Microsomes. (*a*) By ultracentrifuging. The supernatants of tissue suspensions centrifuged at 12 000 *g* for 10 min. were spun in a model L Spinco preparative centrifuge (no. 40 rotor) for 50 min. at 105 000 $g_{av.}$. The microsome pellets were washed with 1 ml. of medium and suspended in 0·4 ml. of medium/g. of original tissue for liver, and in 0·4 ml. of medium/2 g. of original tissue for spleen, bone marrow and lymphosarcoma, by gentle homogenization. (*b*) By aggregation with Mg^{2+} ions. The supernatants of tissue suspensions in 0·35M-sucrose centrifuged at 12 000 *g* for 10 min. were brought to a $MgCl_2$ concentration of 0·02M and the aggregated microsomes were centrifuged for 10 min. at 12 000 *g*. The pellet was washed with a solution containing 0·35M-sucrose and 0·02M-$MgCl_2$, and was suspended by gentle homogenization in the medium in which the $MgCl_2$ was replaced by water, 0·4 ml. of the medium being used for 1 g. of original tissue wt. of liver, or 2 g. of spleen and lymphosarcoma.

(3) Mitochondria + microsomes. The supernatants of tissue suspensions centrifuged at 5000 *g* for 10 min. were spun in a model L Spinco preparative centrifuge (no. 40 rotor) for 50 min. at 105 000 $g_{av.}$. The pellet containing mitochondria + microsomes was washed with 1 ml. of medium and suspended by gentle homogenization in 0·8 ml. of medium/g. of original tissue for liver, and in 0·8 ml. of medium/2 g. of original tissue for spleen.

(4) Cell sap. Liver and spleen were homogenized in 2·5 vol. of 0·25M-sucrose and centrifuged for 50 min. in a model L Spinco preparative centrifuge (no. 40 rotor) at 105 000 $g_{av.}$. The supernatant was carefully pipetted out and used without dilution.

(5) pH 5 Fraction. The cell sap freed of microsomes either by ultracentrifuging or by $MgCl_2$ was brought to pH 5·2 by the addition of M-acetic acid. The precipitate ('pH 5 fraction') was centrifuged and suspended in one-fifth of the original volume of medium.

Method of incubation. The cell-free suspensions of isolated fractions were incubated under a continuous stream of $O_2 + CO_2$ (95:5) or $N_2 + CO_2$ (95:5) at 37° for 30 min. in a total volume of 1 ml. When mitochondria were present the incubation mixture contained 1 μmole of ATP, 1 μmole of FDP, 1 μmole of nicotinamide, mitochondria and labelled amino acid. When mitochondria were absent the incubation mixture contained 1 μmole of ATP, 10 μmoles of PEP and labelled amino acid. Where the cell sap was replaced by the pH 5 fraction 0·25 μmole of GTP and 0·05 ml. of PEP kinase solution were also added.

Isolation of proteins. If, after incubation, it was necessary to separate the different fractions the mitochondria were separated by centrifuging for 10 min. at 12 000 *g*, microsomes for 50 min. at 105 000 *g* and other particles ('post-microsomes') 180 min. at 105 000 *g*. The proteins were precipitated with aqueous 5% (w/v) trichloroacetic acid. They were then treated with hot trichloroacetic acid and organic solvents as described by Zamecnik, Loftfield, Stephenson & Steele (1951). In some cases, the normal washing procedure was followed by treatment of the protein with thioglycollic acid as reported by Siekevitz (1952) under the experimental conditions described by Campbell & Greengard (1959).

Measurement of radioactivity. The proteins were counted at infinite thickness on 0·28 cm.^{2} stainless-steel disks in a Tracerlab SC-16 windowless flow counter. The protein was compressed on the disk with a stainless-steel pestle as described by Busch, Simbonis, Anderson & Greene (1956). The counting procedure was as previously described (Campbell & Greengard, 1959).

RESULTS

Incorporation of different amino acids into nuclei-free suspensions

In metabolic studies on subcellular particles it is important to ensure that the preparations used do not contain whole cells. This is of particular importance with lymphatic tissues, in which the cells are difficult to disrupt. For this reason whole-tissue homogenates have not been employed in the present experiments. In every case the tissue suspension was first centrifuged at 5000 *g* for 10 min. and the sediment, which will also contain the nuclei, was rejected. This procedure also has the advantage of eliminating red blood cells present in the suspensions. Suspensions treated in this way are described as nuclei-free. The energy required for the incorporation of amino acids in this kind of tissue suspension containing mitochondria may be provided either by the intermediates of the Krebs cycle (see Siekevitz, 1952) or by intermediates in the glycolytic pathway (see Zamecnik & Keller, 1954). Since Wagle *et al.* (1958) used FDP, with spleen preparations, the same compound has been used in the present experiments. In preliminary experiments it was found that some of the conditions reported by Wagle *et al.* were not optimum for the incorporation of radioactivity. The incorporation was higher when the concentration of FDP was increased from 0·1 to 1·0 mM in the suspension and by the addition of mM-nicotinamide (see Zamecnik & Keller, 1954). The addition of coenzyme I, used by Emmelot (1957), did not enhance the incorporation. It can be seen from Table 1 that the suspensions prepared from the different lymphatic tissues, and from liver, incorporate labelled amino acids into their proteins by a mechanism which is stimulated by the addition of an energy source. With lymphosarcoma the effect of FDP was very small; the reason for this

small effect of FDP is not known. When nuclei-free suspensions were prepared from lung there was no significant incorporation of amino acids.

It will be seen that the activity of the spleen preparation for the incorporation of leucine was less than that of the liver preparation. In contrast Wagle *et al.* (1958) found that the incorporation of alanine, methionine and phenylalanine respectively was similar in preparations of spleen and liver. This suggested that a comparison of the incorporation of a range of different amino acids in preparations from the two types of tissue might be of interest. The results of such a comparison are shown in Table 2. It will be seen that the amino acids studied can be divided into two groups: in the first, containing glycine, alanine and glutamic acid, the incorporation in the presence of FDP into spleen is at least as great as into liver, whereas in the second group, containing lysine and leucine, the incorporation in the presence of FDP is much greater into liver than into spleen preparations.

Since glycine was used as one of the radioactive amino acids it was thought necessary to exclude the possibility that the results were affected by the synthesis of glutathione or similar peptides which could be bound to the proteins through –S–S– linkages. For this reason, the radioactive proteins were extracted with thioglycollic acid. As was found previously (Campbell & Greengard, 1959), this had no effect on the radioactivity of the proteins when leucine and lysine were used but considerably reduced the count for glycine. Wagle *et al.* (1958) used GTP in their experiments on the incorporation of amino acids into spleen suspensions. Keller & Zamecnik (1956) reported that in liver GTP is present in the cell sap and is needed only when purified preparations of the cell sap are used. It is still possible that spleen suspensions do not contain optimum amounts of GTP, but in our experiments reported in Table 2 the presence of GTP inhibited the incorporation of each of the different amino acids tested.

It can be seen from Table 2 that the incorporation of lysine into spleen suspensions is inhibited by the presence of FDP. The high control value in this case suggested that lysine was being incorporated by a non-energy-requiring mechanism, as has previously been observed (Borsook, Deasy, Haagen-Smit, Keighley & Lowy, 1950; Sarkar, Clarke & Waelsch, 1957), and it seemed possible that spleen contained a very active system for this type of incorporation. These authors have shown that the microsomes are not necessary for the incorporation of lysine into the proteins of the cell sap. Two separate enzyme systems appear to be involved in the incorporation, one which is enhanced by the presence of Ca^{2+} ions and inhibited by the presence of PO_4^{3-} ions and the other which is unaffected by the presence of these ions. It may be seen from Table 3 that when the cell sap is incubated with [^{14}C]lysine there is some incorporation of radioactivity into both tissue preparations, but that spleen is very much more active in this respect than liver. Whereas the incorporation into liver-cell sap was enhanced by the presence of Ca^{2+} ions, that into spleen-cell sap was unaffected. The presence of phosphate in the incubation mixture did not affect the incorporation into spleen-cell sap. The results of the experiments with [^{14}C]glutamic acid

Table 1. *Incorporation of labelled amino acids into the proteins of nuclei-free suspensions of liver, spleen, bone marrow and lymphosarcoma*

A sample (0·7 ml.) of the 5000*g* supernatant (see Materials and Methods section) was incubated aerobically for 30 min. with 1 μmole of ATP, 1 μmole of nicotinamide and 1 μc of labelled amino acid, with and without 1 μmole of FDP. An equal volume of 10% trichloroacetic acid was then added. The results are expressed as radioactivity of the protein in counts/min. at infinite thickness/0·28 cm.^{2}.

Tissue	Labelled amino acid	Control	Plus FDP
Liver	Leucine	35	444
Spleen	Leucine	14	60
Bone marrow	Alanine	7	47
Lymphosarcoma	Leucine	37	51

Table 2. *Incorporation of various labelled amino acids into the proteins of nuclei-free suspensions of spleen and liver*

For incubation conditions and expression of the results see Table 1. Where GTP was added to the incubation mixture it was used as described in the Materials and Methods section. The proteins were treated with thioglycollic acid as described in the Materials and Methods section before their radioactivity was determined.

	Liver		Spleen		
Amino acid	Control	Plus FDP	Control	Plus FDP	Plus FDP plus GTP
Glycine	2	41	6	46	8
Alanine	7	71	48	68	28
Glutamic acid	14	27	19	67	25
Lysine	52	540	160	92	—
Leucine	91	381	32	82	73

and [^{14}C]leucine are included to show that no incorporation took place when these amino acids replaced lysine.

Effect of fructose diphosphate on the extent of incorporation

In the experiments reported by Zamecnik & Keller (1954) with liver suspensions the concentration of FDP used was 10 mM, whereas in the experiments discussed so far mM-FDP was used. An increase in the concentration of FDP from 1 to 10 mM doubled the incorporation of [^{14}C]alanine into liver preparations, but did not affect the incorporation of [^{14}C]leucine into spleen preparations (see Table 4). Table 4 also shows that, when such tissue preparations were incubated anaerobically with PEP as energy source, the incorporation of radioactivity was considerably less than with FDP under aerobic conditions.

Table 3. *Incorporation of radioactivity into proteins during the incubation of cell sap with* [^{14}C]*amino acids*

A sample (0·7 ml.) of the cell sap prepared as described in the Materials and Methods section was incubated anaerobically for 30 min. at 37°, with 1 μc of the labelled amino acid, and the final volume brought to 1 ml. with water. Ca^{2+} ions were added as $CaCl_2$ and phosphate as phosphate buffer, pH 7·6. In control experiments the reaction was stopped before incubation and the radioactivity of the proteins determined. Values obtained were subtracted from the experimental results to give the results in the table. Results are expressed as in Table 1.

Amino acid	Additions (20 mM)	Liver	Spleen
Lysine	—	8	42
Lysine	Ca^{2+} ions	32	47
Lysine	Phosphate	—	36
Leucine	Ca^{2+} ions	3	0
Glutamic acid	Ca^{2+} ions	0	1

Table 4. *Energy requirements for the incorporation of labelled amino acids into nuclei-free suspensions of liver and spleen*

A sample (0·7 ml.) of the 5000 *g* supernatants (prepared as described in the Materials and Methods section) was incubated with 1 μmole of ATP, 1 μc of labelled amino acid, [^{14}C]alanine for liver or [^{14}C]leucine for spleen and the additions indicated in the Table. In the presence of nicotinamide the incubation was carried out in an atmosphere of O_2+CO_2 (95:5) and in the presence of PEP the atmosphere was N_2+CO_2 (95:5). For expression of the results see Table 1.

Additions	Liver alanine	Spleen leucine
mM-Nicotinamide	6	10
mM-Nicotinamide plus mM-FDP	20	33
mM-Nicotinamide plus 10 mM-FDP	43	37
10 mM-PEP	17	17

Distribution of radioactivity in the subcellular fractions

Siekevitz (1952) has shown that when suspensions of disrupted liver cells are incubated with [^{14}C]-alanine and the various subcellular fractions are isolated by centrifuging the most active fraction for the incorporation of radioactivity is the microsomes. Siekevitz also found evidence for the aerobic formation of an 'activated alanine' by the mitochondria, which was transferred anaerobically to the microsomes. It was necessary therefore to determine whether the differences in the relative incorporation of the various amino acids into the protein of the tissue suspensions of the spleen and

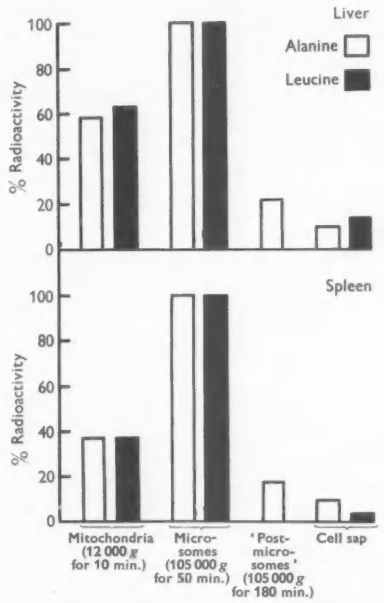

Fig. 1. Distribution of radioactivity in the different subcellular fractions of liver and spleen after incubation of suspensions in the presence of [^{14}C]alanine and [^{14}C]-leucine. The supernatant (2·1 ml.) obtained by centrifuging the liver suspension at 5000 *g* was incubated with 3 μc of labelled amino acid, 3 μmoles of FDP and 3 μ-moles of nicotinamide. A similar supernatant (3·5 ml.) from spleen was incubated with 5 μc of labelled amino acid, 5 μmoles of FDP and 5 μmoles of nicotinamide. After incubation for 30 min. under O_2+CO_2 (95:5) the various subcellular fractions were isolated as described in the Materials and Methods section. The proteins in the fractions were precipitated with 5% trichloroacetic acid and their radioactivity was determined. Results have been expressed as a percentage of the radioactivity of the microsomal fraction for each amino acid.

liver shown in Table 2 were due to differences in the intracellular distribution of the amino acid incorporation. In this and subsequent experiments alanine has been used as an example of the first group of amino acids and leucine of the second group. The results of the experiment shown in Fig. 1 indicate that in both tissues the incorporation of each amino acid occurs to the same extent in the microsomal and mitochondrial fractions. The incorporation of the amino acids into mitochondria compared with that of the microsomes is relatively less in spleen than in liver. The small particles that are sedimented on prolonged centrifuging, i.e. 'post-microsomes', are relatively inert compared with the microsomes. The specific activities of the microsomal proteins (respectively for liver 896 counts/min. with leucine, 75 with alanine; for spleen 366 with leucine, 369 with alanine) show again that whereas in spleen no great differences could be found for the extent of incorporation of the two labelled amino acids, a tenfold incorporation was found in liver with leucine instead of alanine.

Effect of cell sap on the incorporation of labelled alanine

As the mitochondrial fraction did not seem to be responsible for the differences of incorporation of alanine and leucine into liver preparations, the possibility that the cell sap could be responsible was considered. The incorporation of alanine has been followed with particulate fractions (mitochondria plus microsomes) of liver and spleen in the presence of cell sap from the two tissues. It has been found that the incorporation into liver proteins is enhanced when liver-cell sap is replaced by that of spleen (see Table 5), suggesting a greater incorporating ability of the spleen soluble cytoplasm for this amino acid. On the other hand, whereas spleen particles in the presence of spleen-cell sap fail to show an energy-dependent incorporation, these particles in the presence of liver-cell sap actively incorporate alanine.

Incorporation into nuclei- and mitochondria-free suspensions

The use of tissue suspensions freed of mitochondria by centrifuging of the tissue homogenate at 12 000 *g* for 10 min. was introduced by Zamecnik & Keller (1954), using liver suspensions. In the original experiments creatine phosphate was used as the energy donor, but in later experiments other energy-donor compounds such as PEP, 3-phosphoglyceric acid or carbamyl phosphate were used. Although spleen preparations were active also with 3-phosphoglyceric acid the experiments have been carried out with PEP as an energy donor. All the tissues used were found to incorporate labelled amino acids depending on the presence of an energy-donor compound (see Table 6). Again a similar preparation of lung failed to incorporate actively labelled alanine. In such systems it may be observed that whereas liver suspensions incorporate leucine more actively than a similar spleen suspension much smaller differences in the extent of incorporation are found with alanine as the labelled amino acid.

Incorporation into microsomal preparations

It seemed possible that the differences in the incorporation of alanine and leucine in the subcellular suspensions of spleen and liver could have been due to the presence of different amounts of the free amino acids in the preparations of the two tissues. The radioactive amino acids added to the incubation mixtures would then have been diluted to a different extent in the two cases, although the results reported in Table 5 made this explanation unlikely. In order to clarify the position it was desirable to use isolated microsome preparations with purified cell sap.

Table 5. *Incorporation of* [^{14}C]*alanine in liver and spleen subcellular particles (microsomes and mitochondria) in the presence of spleen- and liver-cell sap*

A suspension of the particles (0·4 ml.; prepared as described in the Materials and Methods section) was incubated for 30 min. at 37° with 0·3 ml. of the cell sap, 1 μmole of ATP, 1 μmole of nicotinamide and 1 μc of [^{14}C]alanine in the presence and absence of 1 μmole of FDP. For the expression of results see Table 1.

	Liver-cell sap		Spleen-cell sap	
Particles	Control	Plus FDP	Control	Plus FDP
Liver	19	41	44	103
Spleen	15	42	20	22

Table 6. *Incorporation of labelled amino acid into proteins of various tissue suspensions freed of nuclei and mitochondria*

A sample (0·7 ml.) of a 12 000 *g* supernatant (prepared as described in the Materials and Methods section) was incubated in an atmosphere of $N_2 + CO_2$ (95:5) for 30 min. at 37° with 1 μmole of ATP and 1 μc of labelled amino acid, with and without 10 μmoles of PEP. For the expression of the results see Table 1.

	Leucine		Alanine	
Tissue	Control	Plus PEP	Control	Plus PEP
Liver	3	331	6	31
Spleen	7	99	0	35
Bone marrow	—	—	14	57
Lymphosarcoma	17	115	—	—

Table 7. *Incorporation of labelled amino acids into microsomes plus cell sap or microsomes aggregated by Mg^{2+} ions plus* pH 5 *fraction, from different tissues*

In the experiments with the microsomes plus cell sap, the incubation mixture contained 0·4 ml. of microsome suspension and 0·3 ml. of cell sap, 1 μmole of ATP and 1 μc of labelled amino acid with and without PEP. In the experiments with the microsomes aggregated by Mg^{2+} ions plus pH 5 fraction, the incubation mixture contained 0·4 ml. of suspension of microsomes aggregated by Mg^{2+} ions, 0·3 ml. of pH 5 fraction, 1 μmole of ATP, 0·25 μmole of GTP and 2·5 μmoles of reduced glutathione, with and without 10 μmoles of PEP and PEP-kinase. For the expression of the results see Table 1.

	Microsomes prepared by ultracentrifuging plus cell sap				Microsomes prepared by aggregation with Mg^{2+} ions plus pH 5 fraction			
	Alanine		Leucine		Alanine		Leucine	
	Control	Plus PEP	Control	Plus PEP	Control	Plus PEP	Control	Plus PEP
Liver	12	134	6	1940	7	56	49	346
Spleen	30	155	78	425	10	46	22	50
Lymphosarcoma	16	48	12	109	15	32	17	42
Bone marrow	13	16	22	40	—	—	—	—

Table 8. *Incorporation of labelled alanine and leucine into microsome plus cell sap from liver and spleen*

The incubation mixture contained 0·4 ml. of microsome suspension, 0·3 ml. of cell sap, 1 μmole of ATP and 1 μc of labelled amino acid with and without 15 μmoles of PEP in the final volume of 1 ml. The incubation was carried out for 30 min. at 37° under an atmosphere of $N_2 + CO_2$ (95:5). For the expression of the results see Table 1.

	Liver-cell sap		Spleen-cell sap	
Microsomes	Control	Plus PEP	Control	Plus PEP
	Alanine			
Liver	12	134	21	214
Spleen	28	86	30	155
	Leucine			
Liver	6	1940	9	1734
Spleen	8	193	78	425

Microsomes were isolated from homogenates of both liver and various lymphatic tissues by ultracentrifuging. When such preparations were incubated with the cell sap derived from the tissue of origin an energy-dependent incorporation of radioactivity was obtained, as shown in Table 7. The preparations of bone marrow were less active than those of the other tissues and the incorporation of alanine in the extract from this tissue was not stimulated by the addition of PEP.

An alternative method for the preparation of active microsomes was provided by the work of Siekevitz (1952) and Gjessing, Floyd & Chanutin (1951). These authors showed that microsomes could be caused to aggregate by the addition to the tissue suspension of Mg^{2+} ions. Hoagland, Keller & Zamecnik (1956) and Keller & Zamecnik (1956) have shown that the fraction of the cell sap which is precipitated at pH 5·2 contains all the substances, with the exception of GTP, required for the incorporation of amino acids into microsomal proteins in the presence of an energy-generating system. Such a fraction has been incubated with the microsomes aggregated by Mg^{2+} ions and, as shown in Table 7, an energy-dependent incorporation of amino acid was obtained.

It may be observed that whereas the incorporation of [^{14}C]alanine is similar for both preparations from spleen and liver, the extent of incorporation of [^{14}C]leucine is very much greater in the preparations from liver than from spleen.

Effect of cell sap on the incorporation of amino acids into microsomes

As the incorporation of alanine into liver particles (mitochondria + microsomes) was greater in the presence of spleen-cell sap than liver-cell sap (Table 5), similar experiments have been carried out with microsomes obtained by ultracentrifuging from liver and spleen, alanine or leucine being used separately. In Table 8 it is seen that, if incorporation in the presence of PEP is considered, there is more incorporation with liver microsomes in the presence of spleen-cell sap than in the presence of liver-cell sap. However, with spleen microsomes, there is more incorporation in the presence of spleen-cell sap than in the presence of liver-cell sap. With labelled leucine and liver microsomes there is slightly less incorporation in the presence of spleen-cell sap than liver-cell sap, and with spleen microsomes there was more incorporation in the presence of spleen-cell sap than liver-cell sap.

The cell sap is a complex mixture of substances and it is possible that some compounds interfering with the stability of the microsomes could be present. It was therefore thought desirable to repeat these experiments with the partially purified cell sap (pH 5 fraction) instead of the whole-cell sap. The results are shown in Table 9. In this case, with either [^{14}C]leucine or [^{14}C]alanine the incorporation into both liver and spleen microsomes was greater in the presence of the pH 5

Table 9. *Incorporation of labelled alanine and leucine into microsomes plus* pH 5 *fraction from liver and spleen*

The incubation mixture contained 0·4 ml. of microsome suspension, 0·3 ml. of the pH 5 fraction, 1 μmole of ATP, 0·25 μmole of GTP and 1 μc of labelled amino acid, with and without 15 μmoles of PEP and PEP-kinase. Incubation was for 30 min. at 37° under an atmosphere of $N_2 + CO_2$ (95:5). For the expression of the results see Table 1.

	Liver pH 5 fraction		Spleen pH 5 fraction	
Microsomes	Control	Plus PEP	Control	Plus PEP
		Alanine		
Liver	8	312	5	87
Spleen	6	36	2	11
		Leucine		
Liver	15	1828	8	997
Spleen	10	181	10	125

fraction of liver than that of spleen. The spleen pH 5 fraction had a very low activity for the incorporation of [^{14}C]alanine into spleen microsomes.

DISCUSSION

In any comparison of the extent of incorporation of amino acids by subcellular fractions of different tissues the isolation of the components of the cell presents a major problem. Since ultrasonic disruption of liver microsomes substantially reduces the ability of the particles to incorporate amino acids (P. N. Campbell, unpublished observations), it is necessary to ensure than in the process of disruption of the cell the endoplasmic reticulum and its ribonucleoprotein particles are not damaged. This being so, it is not surprising that it is difficult to prepare satisfactory suspensions from tissues which contain large quantities of connective tissue, since homogenization must then be prolonged. Among the tissues studied in the present experiments lung and lymphosarcoma present particular difficulties in this respect. In some tissues such as bone marrow it is difficult to disrupt the cells at all.

As was explained above, practical considerations required the removal of the nuclei from the cell suspensions before incubation. A further advantage in this procedure arises from the fact that liver nuclei have been shown to inhibit the incorporation of amino acids into liver microsomes (Campbell & Greengard, 1959). Two types of subcellular preparations have been used therefore: one containing mitochondria plus microsomes plus cell sap incubated anaerobically with FDP and the other containing microsomes plus cell sap incubated anaerobically with PEP.

In the suspensions containing mitochondria the influence of the proportion of mitochondria to the total protein content must be considered. Thus the liver suspensions contained 4·5 mg. of mitochondrial protein/ml. of suspension, whereas spleen contained 1·7 mg./ml., the concentration of total protein in each suspension being similar. On this basis, the liver suspension would be expected to be able to utilize more FDP than that of the spleen. This was, in fact, found to be so.

The hope that the lymphosarcoma, being a rapidly growing tissue, might provide subcellular fractions with a higher activity than normal tissues was not fulfilled. In fact, the mechanism for the incorporation of amino acids by the tumour tissue did not appear to differ from that of the normal lymphatic tissues. Nevertheless the tumour tissue does have the advantage over spleen in that it contains fewer red blood cells, the protein of which would be relatively inert metabolically.

It remains to discuss the significance to be attached to the differences found in the extent of incorporation of leucine and alanine by subcellular preparations of liver and lymphatic tissue. For the purpose of the present discussion, it is assumed that the incorporation of amino acids in these preparations of subcellular particles represents the synthesis of complete protein from amino acids without the mediation of peptides. Provided that the amino acid composition of the synthesized protein in the two different tissues is similar, then the ratio of the specific radioactivity of the free amino acids and the protein-bound amino acids should be independent of the nature of the amino acid being studied (Campbell & Halliday, 1957). The amino acid composition of the protein being synthesized in spleen- and liver-tissue fractions is, of course, not known, but that of the whole-tissue proteins is similar (Müting & Wortmann, 1954).

On the basis of the figures given for the concentration of free alanine and leucine of cat liver by Tallan, Moore & Stein (1954), the specific radioactivity of the free amino acids in the cell sap used in the incubation of the microsomes would be 3·8 c/mole for alanine and 7·1 for leucine. Since the leucine and alanine content of liver protein is similar (Müting & Wortmann, 1954) it seems unlikely that these differences in the specific activity of the precursor amino acids could account for the tenfold difference in the extent of incorporation of the two amino acids by liver preparations.

Estimations of the concentration of free alanine in spleen and liver by Campbell & Stone (1957) can be used to show that the specific radioactivity of the free alanine in the cell sap used for the incubation of the microsomes was 3·3 c/mole for spleen and 3·8 c/mole for liver (a figure which agrees well with that based on the results of Tallan *et al.* 1954). In the experiment in which spleen-cell sap replaced liver-cell sap the incorporation of alanine by liver

microsomes was increased. In view of the similarity in the specific radioactivity of the free alanine in the two cell saps this result cannot be attributed to such a factor. Furthermore, when the whole-cell sap was replaced by the pH 5 fraction, which presumably contains only a small proportion of the free amino acids of the whole-cell sap, the extent of the incorporation of leucine into the spleen and liver microsomes was unaffected.

It may be observed that with spleen microsomes incubated with alanine, replacement of the whole-cell sap by the pH 5 fraction reduced the extent of the incorporation of amino acid. This suggests that a factor required for the incorporation of alanine with spleen-cell sap is not precipitated at pH 5·2.

One further point emerges from the studies with cell sap and pH 5 fraction. When [^{14}C]leucine is incubated with spleen microsomes the incorporation is greater in the presence of spleen-cell sap than of liver-cell sap, whereas liver pH 5 fraction is more effective than that of spleen. It seems probable that in the process of precipitation at pH 5·2 the activity of an inhibitor in the liver-cell sap for the incorporation of leucine is lost.

Although it is not possible at present to understand fully the interplay of the various factors involved in the incorporation of amino acids into subcellular fractions, two conclusions appear to be warranted. First, the ability of the microsome preparations from different tissues to synthesize protein *in vitro* is limited. The extent of this limitation is not necessarily correlated with activity *in vivo*. For example, under optimum experimental conditions, liver microsomes incorporate a given amino acid more actively than spleen microsomes. Secondly, the extent of incorporation of a given amino acid into the microsomes is regulated by factors present in the cell sap. The nature of these regulating factors is the subject of a later paper (Rendi & Campbell, 1959).

SUMMARY

1. When tissue suspensions containing mitochondria and microsomes are prepared from rat spleen, bone marrow and transplanted Murphy lymphosarcoma and are incubated with fructose diphosphate under aerobic conditions, an energy-dependent incorporation of amino acids takes place.

2. Whereas the extent of incorporation of ^{14}C-labelled glycine, alanine and glutamic acid was of the same order in such preparations of liver and spleen, ^{14}C-labelled leucine and lysine were more actively incorporated in the preparations from liver.

3. When [^{14}C]lysine, but not the other amino acids tested, was incubated with spleen-cell sap, the isolated protein was radioactive. The labelling of this protein was only slightly affected by the presence of Ca^{2+} or PO_4^{3-} ions in the medium.

4. When suspensions of lymphatic tissues were freed of mitochondria and incubated under anaerobic conditions with phosphoenolpyruvate, [^{14}C]alanine and [^{14}C]leucine were incorporated.

5. Active microsomes could be obtained from these tissues by either ultracentrifuging or aggregation with Mg^{2+} ions. When the microsomes prepared from spleen or liver were incubated with cell sap and phosphoenolpyruvate, alanine was incorporated to about the same extent in the two preparations whereas leucine was more actively incorporated in the preparation from liver.

6. When liver-cell sap was replaced by spleen-cell sap the incorporation of [^{14}C]alanine into liver microsomes was enhanced whereas the incorporation of [^{14}C]leucine was only slightly affected. On the other hand, the replacement of spleen-cell sap by liver-cell sap reduced the incorporation of both amino acids into spleen microsomes.

7. When the fraction of the spleen-cell sap precipitated at pH 5·2 replaced a similar fraction from liver, the incorporation of both amino acids into spleen and liver microsomes was reduced.

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Transglucosidase Activity of Rumen Strains of *Streptococcus bovis*

2. ISOLATION AND PROPERTIES OF DEXTRANSUCRASE*

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Dextransucrase, the enzyme which converts sucrose into dextran and fructose, has been found in the cell-free culture fluids of various strains of *Leuconostoc mesenteroides* (Hehre, 1951; Tsuchiya *et al.* 1952). The properties of the isolated enzymes have been described in detail (Koepsell *et al.* 1953; Bailey, Barker, Bourne & Stacey, 1957*a*). A number of species of streptococci have also been shown to produce dextran from sucrose (Hehre, 1951). In these organisms, however, the enzyme responsible for dextran synthesis does not appear to have been isolated or examined. Recently it was shown that various strains of *Streptococcus bovis* produce dextran in sucrose cultures only in the presence of added carbon dioxide (Dain, Neal & Seely, 1956; Bailey & Oxford, 1958*a*, *b*). In *Leuconostoc* species, however, dextran production appears to be independent of added carbon dioxide (Bailey & Oxford, 1958*a*). In view of the lack of information on streptococcal dextransucrase and because of the interesting effect of carbon dioxide it was decided to isolate and examine *S. bovis* dextransucrase.

In isolating *Leuconostoc* dextransucrase the main problem has been to obtain a preparation of the enzyme entirely free from preformed dextran. Various authors (e.g. Tsuchiya *et al.* 1952; Neely, 1958) have used the cell-free culture fluid from a *L. mesenteroides* strain grown in 2% sucrose medium as a dextransucrase preparation. Bailey *et al.* (1957*a*) found that dextransucrase isolated from this type of culture contained much dextran and could not be freed from this dextran. A modified method (Bailey, Barker, Bourne & Stacey, 1957*b*) gave a dextransucrase containing much less dextran. In this case, however, the enzyme had only weak dextransucrase activity except in the presence of added acceptor sugar, when it produced oligosaccharides and very little dextran.

This paper describes the isolation and properties of *S. bovis* dextransucrase, including a comparatively dextran-free preparation which gave a good conversion of sucrose into dextran and fructose.

* Part 1: Bailey (1959).

EXPERIMENTAL

Organism. *S. bovis* (strain I; Bailey & Oxford, 1958*a*, *b*), isolated from the rumen of a cow feeding on red clover, was used.

Culture media. (1) Basal medium. The organism was normally grown in a Bacto Casitone–Bacto Yeast Extract medium (Bailey & Oxford, 1958*b*) containing either sucrose (A.R.; 4%, w/v) or glucose (A.R.; 2%, w/v). Cultures were maintained in both the sucrose and glucose media.

(2) Synthetic medium. Oxford (1958) developed a synthetic medium suitable for growth and dextran production with *S. bovis*. This medium contained the same concentration of sucrose or glucose as the basal medium. Both basal and synthetic media were adjusted to pH 7·0. All cultures were incubated at 37° in CO_2 unless stated otherwise.

Carbohydrates. Sucrose and glucose were A.R. grade. All sugars used were shown, by paper-chromatographic analysis, to be free from other sugars (less than 0·1% of impurity). Dextran was isolated by precipitation with ethanol (2 vol.) from the cell-free culture fluid of *S. bovis* (strain I) which had been grown on sucrose for 8 hr. (to pH 5·1). The dextran was purified by the usual methods, e.g. Bailey & Oxford (1958*b*). Isomaltose and isomaltotriose were isolated from a partial acid hydrolysate of the dextran by fractionation with aqueous ethanol after absorption on a charcoal–Celite column (Turvey & Whelan, 1957).

Paper chromatographic analyses. Solutions were analysed for sugars by paper chromatography with the solvents and sprays described in Part 1 (Bailey, 1959). An additional spray, naphthoresorcinol (Forsyth, 1948), was employed. Enzyme solutions were desalted by treatment with Dowex 2 and Dowex 50 ion-exchange resins.

Standard method for measuring dextransucrase activity. Experiments showed that *S. bovis* dextransucrase acted on sucrose in the same manner as the *Leuconostoc* enzyme. The method used for measuring the activity of *Leuconostoc* dextransucrase (Bailey *et al.* 1957*a*), which depends on the measurement of the liberated fructose in an enzyme–sucrose solution, was therefore used for measuring the activity of *S. bovis* dextransucrase. The only modification to the method was that the solutions for measurement of activity were incubated at 37°. The solutions (7 ml.) contained either freeze-dried enzyme (2–5 mg.) or cell-free culture fluid (1–2 ml.), sucrose (300 mg.) and acetate buffer (0·05 M, pH 5·2; 5–7 ml.). Fructose was measured by the method of Shaffer & Hartmann (1921) after treatment with hypoiodite (Van der Plank, 1936). Controls showed that fructose was liberated only in the solutions containing both dextransucrase and sucrose. A unit of dextransucrase activity (*Leuconostoc* enzyme) has been defined as the level of enzyme activity which converts 1 mg. of sucrose into fructose and dextran in 1 hr. at a specified temperature and pH, provided that not more than half of the sucrose has been consumed (Koepsell & Tsuchiya, 1952). This definition was used in the present work. As with the *Leuconostoc* enzyme (Bailey *et al.* 1957*a*) high concentrations of fructose depressed the activity of *S. bovis* dextransucrase. However, the conditions used in measuring dextransucrase activity were such that the amount of fructose produced never approached the concentration at which significant depression of enzyme activity occurred. In measuring the activity of undialysed cell-free cultures a correction was made for any fructose already present. Minor modifications made to the standard method when measuring the effect of pH, temperature and added sugars on dextransucrase activity are described below.

Isolation of dextransucrase. S. bovis cultures grew very rapidly and soon reached the range pH 4·3–4·6, where dextransucrase is unstable. Experiments showed that a culture contained its maximum dextransucrase activity when the pH had fallen to between 5·8 and 5·2. With basal medium this generally required incubation for 7–9 hr. at 37°.

Basal medium (250–500 ml.; containing sucrose or glucose) was inoculated from the appropriate *S. bovis* culture and incubated until the pH had dropped to 5·8–5·2. Only small inocula (1–2 drops/250 ml. or one loopful/50 ml.) were used. The final culture was rapidly cooled to 0–2° and centrifuged (30 000 *g*) for 30 min. on a Spinco preparative ultracentrifuge. The enzyme was precipitated from the cell-free supernatant with ammonium sulphate (A.R.; 60 g./100 ml.) and the solution left overnight at 2°. The precipitate was collected by centrifuging (30 000 *g*) at 0–5° for 30 min., dissolved in citrate buffer (0·05 M, pH 6·0; 50 ml.) and recentrifuged. The enzyme solution was dialysed for 48 hr. at 2° against three changes of citrate buffer (2 l.) in dialysis tubing which had been well washed with the citrate buffer. The final solution of enzyme in citrate buffer was recentrifuged and freeze-dried. The polysaccharide content of the freeze-dried dextransucrase preparations was measured by the anthrone method of Roe (1954) and expressed as dextran.

RESULTS

Dextransucrase production during growth of Streptococcus bovis

In order to establish the cultural conditions for maximum dextransucrase production the dextransucrase activity of a growing culture of *S. bovis* was examined at intervals. Basal sucrose medium was inoculated from a sucrose culture of *S. bovis* and incubated at 37°. At intervals portions (20 ml.) were removed and, after measurement of pH, centrifuged (30 000 *g*) at 0–5° for 30 min. Dextransucrase activity in the cell-free fluid was measured by the standard method. The results obtained are listed in Table 1.

The effect of cultural conditions on dextransucrase production by *S. bovis* was examined by measuring the dextransucrase activity in cultures in various media which had been grown to within the range pH 5·8–5·1. The results obtained are listed in Table 2. Cultures in the synthetic medium showed a rather variable lag phase (8–12 hr.) before any growth occurred. These cultures were first incubated overnight at 28°, during which time the pH dropped to 6·0–6·2, and then transferred to the incubator at 37°, when incubation for 2–4 hr. gave a culture within the desired pH range.

Dextransucrase preparations

Two main dextransucrase preparations were isolated and used in an examination of the properties of the enzyme. These were: (1) Dextransucrase I from basal sucrose media (250 ml.) inoculated from a sucrose culture. The culture, after incubation for 8 hr. to pH 5·5, yielded a cell-free culture fluid (200 ml.) containing 5·5 units of dextransucrase/ml. (1100 units). The dialysed

Table 1. *Dextransucrase production in a culture of* Streptococcus bovis

Organism (strain I) was inoculated into basal sucrose (4 %, w/v) medium (200 ml.) and incubated at 37° in CO_2. Portions (20 ml.) were removed at intervals and centrifuged before measurement of dextransucrase activity.

Time of incubation (hr.)	pH of culture fluid	Dextransucrase activity of cell-free culture fluid (units/ml. at pH 5·2)
0	7·0	0
5·5	6·7	0
6·5	6·4	0·1
7·5	5·1	6·2
9·5	4·4	1·75

Table 2. *Dextransucrase activity of various cultures of* Streptococcus bovis

Cultures (50 ml.) containing glucose (2%, w/v) or sucrose (4%, w/v) were incubated at 37° in CO_2 until the pH fell to within the range 5·1–5·8 and were then centrifuged.

Culture medium		pH of culture fluid	Dextransucrase activity of cell-free culture fluid (units/ml. at pH 5·2)
Inoculation	Test		
Basal, sucrose	Basal, sucrose	5·3	5·1
Basal, sucrose	Basal, sucrose (no CO_2)	5·3	1·0
Basal, sucrose	Basal, glucose	5·1	3·7
Basal, glucose (sixth successive culture)	Basal, glucose	5·8	2·4
Synthetic, sucrose	Synthetic, sucrose	5·4	0·5
Synthetic, glucose (sixth successive culture)	Synthetic, glucose	5·4	0·2

solution of the ammonium sulphate precipitate contained 800 units and the final freeze-dried solid (0·7 g.) 790 units of dextransucrase activity. (2) Dextransucrase II from basal glucose medium (500 ml.) inoculated from a sucrose culture. The cell-free culture fluid (475 ml.) obtained from the culture after incubation for 8 hr. to pH 5·2 contained 3·15 units of dextransucrase activity/ml. (1500 units). The final dialysed solution contained 1300 units of activity and the freeze-dried solid (1·12 g.) 1240 units of activity.

Dextransucrase I contained 70% of polysaccharide (0·62 mg./unit of dextransucrase activity) and 1·79% of nitrogen. It gave in water a solution with the opalescence typical of a dextran solution. Dextransucrase II, however, contained only 4% of polysaccharide (0·036 mg./unit of dextransucrase activity). This enzyme preparation contained 9·06% of nitrogen. It gave a clear solution in water. Other preparations of dextransucrase isolated from glucose cultures contained similar low concentrations of polysaccharide.

Action of dextransucrase on sucrose and other carbohydrates

Both enzyme preparations were incubated with sucrose and tested for dextransucrase activity. Solutions were incubated for 3 and 24 hr. together with appropriate controls containing (*a*) dextransucrase but no sucrose, (*b*) boiled dextransucrase and sucrose and (*c*) sucrose but no enzyme. Paper chromatograms of the desalted solutions at the end of incubation showed no free sugars in the controls except for added sucrose. The solutions with enzyme plus sucrose, however, showed in addition to sucrose a component chromatographically identical with fructose; glucose was absent from the papers. The intensities of the fructose spots on the papers were proportional to the time of incubation. The two enzymes produced dextran, as shown by the development of opalescence. The enzyme preparations always produced fructose plus polysaccharide but no glucose from sucrose. No sign of invertase activity was detected in cell-free culture fluids from the various cultures, including those grown in sucrose medium in the absence of carbon dioxide.

Table 3. *Effect of sucrose concentration on dextransucrase activity*

Solutions (7 ml.) containing dextransucrase (2 mg.), acetate buffer (0·05 M, pH 5·2; 5 ml.) and sucrose were incubated at 37° for 3 hr.

Sucrose (mg.)	Dextransucrase activity (units)	
	Dextransucrase I	Dextransucrase II
25	1·52	1·52
50	1·90	1·81
100	2·03	2·06
200	2·28	2·23
300	2·28	2·23

The effect of sucrose concentration on dextransucrase activity was examined by measuring the fructose liberated, after incubation for 3 hr., in solutions containing dextransucrase (2 mg.) and various amounts of sucrose. The results, converted into units of dextransucrase activity, for the two dextransucrase preparations are listed in Table 3. A similar effect of sucrose concentration on enzyme activity has been reported for *Leuconostoc* dextransucrase (Bailey *et al.* 1957*a*).

The effect of incubation time on dextransucrase activity was examined by incubating solutions containing dextransucrase (2 mg.) and sucrose (500 mg.) for various times and measuring the liberated fructose. The results obtained, for both enzyme preparations, are listed in Table 4. On prolonged incubation there is an apparent lowering of dextransucrase activity due, in part at least, to the depressing effect of high fructose concentrations on dextransucrase activity (see Table 6). In the standard method used for measuring dextransucrase activity, solutions are incubated for only 3 hr., when no decrease in activity occurs.

Table 4. *Effect of incubation time on dextransucrase activity*

Solutions (7 ml.) containing dextransucrase (2 mg.), sucrose (500 mg.) and acetate buffer (0·05 M, pH 5·2; 5 ml.) were incubated at 37°.

Incubation time (hr.)	Dextransucrase I		Dextransucrase II	
	Fructose produced (mg.)	Dextransucrase activity (units)	Fructose produced (mg.)	Dextransucrase activity (units)
3	3·6	2·28	3·5	2·23
6	7·2	2·28	7·0	2·23
17	18·3	2·05	18·1	2·03
24	23·8	1·88	24·0	1·90
48	35·3	1·40	37·1	1·47

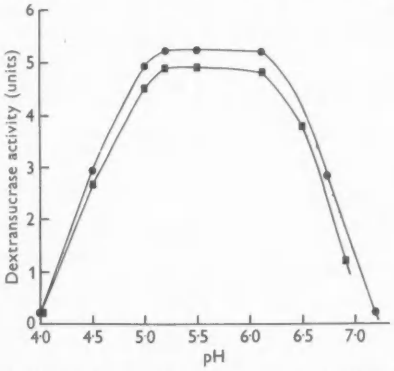

Fig. 1. Effect of pH on dextransucrase activity. Solutions containing dextransucrase (5 mg.) and sucrose (300 mg.) in acetate solution (0·05 M; 5 ml.) were incubated for 3 hr. at 37°. ●, Dextransucrase I; ■, dextransucrase II.

Carbon dioxide undoubtedly stimulates dextran production in sucrose cultures of *S. bovis* (Bailey & Oxford, 1958*a*, *b*). To test the effect of carbon dioxide on the enzymes, solutions of dextransucrase I and II with sucrose were incubated in unstoppered flasks for 3 hr., in jars filled with either CO_2 or air. Measurement of the liberated fructose showed that the dextransucrase activity was the same in air or CO_2.

The action of the enzyme preparations on other sugars was also checked. Solutions were prepared each containing dextransucrase (I and II; 5 mg.) and fructose or glucose (10 mg.) but no sucrose. The reducing sugar content of each solution was measured both before and after incubation for 24 hr. under toluene. There was no loss of reducing sugar in any of the solutions. Other solutions containing dextransucrase (5 mg.) and various added sugars (20 mg.) but no sucrose were incubated for 24 hr. and the solutions examined by paper-chromatographic analysis. The results obtained showed that both enzyme preparations were without action on maltose, isomaltose, raffinose, melibiose, cellobiose, lactose, $\alpha\alpha$-trehalose, melezitose and starch.

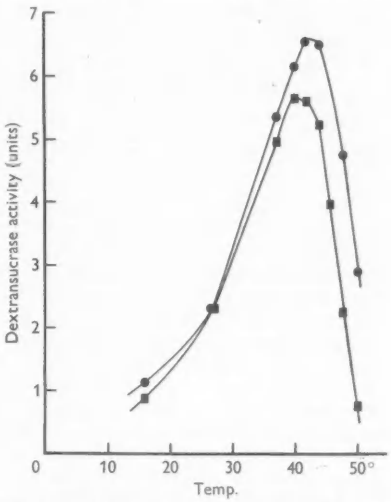

Fig. 2. Effect of temperature on dextransucrase activity. Solutions containing dextransucrase (5 mg.) and sucrose (300 mg.) in acetate buffer (0·05 M, pH 5·2; 5 ml.) were incubated for 3 hr. ●, Dextransucrase I; ■, dextransucrase II.

Effect of pH *and temperature on dextransucrase activity*

The effect of pH on dextransucrase activity was obtained by measuring activity in solutions in which the pH of the added acetate solution (0·05 M) ranged from 4·0 to 7·2. The results obtained with both enzyme preparations, after incubation for 3 hr., are shown in Fig. 1.

The effect of temperature on dextransucrase activity was obtained by measuring the activity in solutions of each enzyme after incubation for 3 hr. at various temperatures. The results obtained are shown in Fig. 2.

Stability of dextransucrase at 37°. The stability of dextransucrase at 37° was examined by incubating dextransucrase II (30 mg.) in acetate buffer (0·05 M, 16 ml.) at pH 4·5 and 5·2, under toluene, in

Table 5. *Stability of dextransucrase solutions at 37°*

Solutions containing dextransucrase II (30 mg.) in acetate buffer (0·05 M, 16 ml.) were stored at 37° under toluene in the presence and absence of dextran. Dextransucrase activities were measured by the standard method at pH 5·2 in 1 ml. portions of the solutions.

pH	Added dextran (mg.)	Dextransucrase activity after storage for 0 hr.	24 hr.	48 hr.	72 hr.
5·2	—	1·96	1·88	1·83	1·80
5·2	100	2·00	2·00	2·00	2·00
4·5	—	2·00	0·14	0	—
4·5	100	2·10	0·59	0·31	—

the absence and presence of dextran. At daily intervals portions (1 ml.) were withdrawn for measurement of dextransucrase activity at pH 5·2. The results, which show the effect of pH and dextran on dextransucrase stability, are given in Table 5.

Effect of added sugars on dextransucrase activity

Koepsell *et al.* (1953) showed that many sugars, in the presence of both *Leuconostoc* dextransucrase and sucrose, either stimulated or depressed dextransucrase activity and, at the same time, acted as alternative glucosyl acceptors to produce oligosaccharides. The effect of added sugars on the activity of *S. bovis* dextransucrase was also examined by the modified standard method for measuring dextransucrase activity as described by Bailey *et al.* (1957*a*). The solutions, which contained enzyme (2 mg.), sucrose (500 mg.) and added sugar (25–300 mg.), were incubated for 24 hr. Appropriate controls were run. The effect of each sugar on dextransucrase activity was expressed as the percentage change in the amounts of fructose liberated relative to the fructose produced in the standard dextransucrase–sucrose solution during incubation for 24 hr. Results from the study of the effect of incubation time on dextransucrase activity (Table 4) show an apparent loss of activity during incubation for 24 hr. In measuring the effect of added sugars on dextransucrase activity the long incubation time is necessary in order to obtain a suitable ratio, for the fructose measurement, of fructose produced to sugar added (Bailey *et al.* 1957*a*). As the results obtained were comparative in nature the falling off in dextransucrase activity during incubation was not considered serious. The results for those sugars (100 mg. dry wt.) which affected dextransucrase activity are listed in Table 6. Mannose, sorbose, raffinose, melezitose, αα-trehalose and dextran (all 100 mg.) had no effect on dextransucrase activity.

Of the readily available sugars, maltose had the most pronounced effect on the activity of *S. bovis* dextransucrase, as was the case with *Leuconostoc* dextransucrase (Koepsell *et al.* 1953; Bailey *et al.* 1957*a*). Dextransucrase activity was measured in

Table 6. *Effect of added sugars on dextransucrase activity*

Solutions containing dextransucrase (2 mg.), sucrose (500 mg.) and added sugar (100 mg.) in acetate buffer (0·05 M, pH 5·2; 7 ml.) were incubated at 37° for 24 hr.

Added sugar	Effect on dextransucrase activity (% change in fructose production) Dextransucrase I	Dextransucrase II
Maltose	+174	+120
Isomaltose	—	+110
Isomaltotriose	—	+55
Glucose	+60	+46
Galactose	+12	+40
D-Arabinose	+12	+33
Methyl α-glucoside	+22	+33
Lactose	+7	+20
Cellobiose	0	+9·2
Melibiose	0	+20
Fructose	0	−29

Table 7. *Effect of added maltose on dextransucrase activity*

Solutions containing dextransucrase (2 mg.), sucrose (500 mg.) and added maltose (anhydrous) in acetate buffer (0·05 M, pH 5·2; 7 ml.) were incubated at 37° for 24 hr.

Maltose (mg.)	Increase in fructose production (%) Dextransucrase I	Dextransucrase II
50	80	60
100	174	120
150	190	143
200	203	174
250	207	188

solutions containing various amounts of added maltose. The results obtained are listed in Table 7.

After desalting with Dowex 2 and Dowex 50 ion-exchange resins the final enzyme solutions and controls were examined by paper chromatography. Prominent series of oligosaccharide spots were present on paper chromatograms of the enzyme solutions containing added maltose, isomaltose and isomaltotriose. Much weaker oligosaccharide spots were present on paper chromatograms of the enzyme solutions containing added glucose, lactose,

cellobiose, fructose, galactose and methyl α-glucoside. None of the other enzyme solutions or any of the control solutions contained any detectable sugar except sucrose, fructose and added sugar.

Enzymically synthesized dextran

Solutions containing dextransucrase (10–15 mg.), sucrose (1 g.) and acetate buffer (0·05 M, pH 5·2; 20 ml.) were incubated for 24 hr. at 37° under toluene. Dextran was precipitated from the opalescent solutions with ethanol (2 vol.), purified and freeze-dried. Three specimens of dextran were synthesized with (1) dextransucrase I (15 mg.), (2) dextransucrase II (15 mg.) and (3) dextransucrase (10 mg.) isolated from a glucose–basal medium culture which had been inoculated with a glucose culture of *S. bovis* (see Table 2). The yields of dextran were 160, 164 and 108 mg. respectively (85–90 % of theoretical). The dextrans did not stain with iodine and dissolved in water to give opalescent solutions. Partial and total acid hydrolysates of each dextran were prepared (Bailey, 1959) and examined by paper chromatography. Total acid hydrolysates of each dextran showed a single component chromatographically identical with glucose. The three partial acid hydrolysates showed components corresponding to glucose and an apparently homologous series of oligosaccharides. The first two members of the series were chromatographically identical with isomaltose and isomaltotriose respectively. The three dextrans had $[\alpha]_D^{20}$ (*c*, 0·15 in N-NaOH) +190°, +191° and +185° respectively.

A small amount of polysaccharide (20 mg.) was synthesized with dialysed cell-free culture fluid from a *S. bovis* culture in synthetic glucose medium (see Table 2). A mixture of cell-free fluid (10 ml.; 2 units of dextransucrase activity) and sucrose (300 mg.) was incubated at 37° for 24 hr. under toluene. The solution became opalescent and polysaccharide was isolated. A partial acid hydrolysate of the polysaccharide showed on paper chromatograms components which were chromatographically identical with glucose, isomaltose and isomaltotriose. A total acid hydrolysate showed a single component chromatographically identical with glucose.

DISCUSSION

S. bovis produces an extracellular enzyme which converts sucrose into a polysaccharide and fructose. The polysaccharide has the properties of dextran and the enzyme is therefore designated a dextransucrase. The action of the enzyme on sucrose, both alone and in the presence of added acceptor sugars, and the effect of sucrose concentration, incubation time and pH on its activity, are similar to the properties of *Leuconostoc* dextransucrase (Bailey *et al.* 1957*a*). Although *S. bovis* produces good yields of dextran only in the presence of carbon dioxide (Bailey & Oxford, 1958*a*, *b*), the isolated dextransucrase is active in its absence. It seems likely therefore that carbon dioxide stimulates either the actual formation of dextransucrase or its release into solution.

The production of dextransucrase in the absence of added sucrose was unexpected and suggests that *S. bovis* dextransucrase might be a constitutive rather than an adaptive enzyme. There are several other possible explanations, including: (1) the glucose media may have contained a trace contamination of sucrose; (2) the organism may secrete traces of intracellular sucrose which are sufficient to stimulate dextransucrase production; (3) *S. bovis* may only gradually cease to produce dextransucrase after it has been deprived of sucrose. In connexion with the first possibility, no sucrose could be detected in a concentrated solution of the yeast extract used in the media or in the glucose medium itself. In a synthetic glucose medium dextransucrase could also be detected although at a much lower level of activity. Good yields of enzyme would not be expected in this glucose medium as results reported by Oxford (1958) suggest that even under optimum conditions of pH, dextransucrase is unstable unless large molecules, either preformed dextran or added protein, are present.

From cell-free culture fluid obtained from a sucrose (2 %, w/v) culture of a *Leuconostoc* species Bailey *et al.* (1957*a*) isolated dextransucrase (containing 70–80 % of dextran) by ethanol precipitation at 0°. When the sucrose cultures also contained maltose (10 %, w/v) (Bailey *et al.* 1957*b*), and produced oligosaccharides rather than dextran, the dextransucrase (containing 7 % of dextran) could be precipitated with ethanol only at −20°. This latter dextransucrase exhibited good activity only in the presence of maltose, when it produced oligosaccharides containing α-1:6-linkages, e.g. panose, and very little dextran. Dextransucrase could not be precipitated with ammonium sulphate from either type of culture. In contrast with these results *S. bovis* dextransucrase is easily precipitated with ammonium sulphate from both sucrose and glucose cultures. Whereas dextransucrase precipitated from sucrose cultures of *S. bovis* contained 70 % of dextran the enzyme from glucose cultures was low in dextran (4 %). This latter enzyme preparation contained only 0·036 mg. of polysaccharide/unit of dextransucrase activity, compared with 1·01 mg. of polysaccharide/unit of dextransucrase activity for the *Leuconostoc* dextransucrase from the sucrose–maltose culture (Bailey *et al.* 1957*b*). Moreover the glucose-culture dextransucrase produced good yields of dextran (90 % of

theoretical) from sucrose. In the dextran specimens prepared with this enzyme only 0·4% of the synthesized dextran was due to the preformed polysaccharide present in the enzyme.

The properties of dextransucrase preparations I and II are in sufficient agreement to suggest that they are the same enzyme. The main difference from *Leuconostoc* dextransucrase is in the effect of temperature on dextransucrase activity. *S. bovis* dextransucrase has optimum activity in the temperature range 37–44° whereas the *Leuconostoc* enzyme has optimum activity between 25° and 29° and rapidly loses activity above 35°. A higher temperature optimum for *S. bovis* dextransucrase is not unexpected, as optimum temperature for growth of the organism is in the range 37–42°. A second possible difference from *Leuconostoc* dextransucrase is the absence of enzymes producing branching of the dextran molecule. Many, but not all, strains of *L. mesenteroides* produce dextrans which are highly branched. The strain of *L. mesenteroides* used by Bailey *et al.* (1957*a*, *b*) produced a dextran containing 10–15% of branch (α-1:3)-linkages (Barker, Bourne, Bruce, Neely & Stacey, 1954). Dextransucrase isolated from cultures of this organism contained, in addition to the enzyme synthesizing the main chain of α-1:6-linked anhydroglucose units, a second, more labile, enzyme which synthesized the branch linkages in the dextran molecule (Bailey *et al.* 1957*a*). Dextran produced by *S. bovis* (strain I) has been shown to be entirely unbranched (Bailey, 1959). The dextransucrase produced by this organism would therefore be expected to be free from branching enzymes and in fact specimens of dextran synthesized with the enzyme have $[\alpha]_D$ values expected for an unbranched dextran.

It is evident that, in addition to any stabilizing effects due to co-precipitated protein or dextran, pH is an important factor in determining the stability of *S. bovis* dextransucrase (see Table 5). The activity of *S. bovis* dextransucrase is stimulated by the same sugars which stimulate the activity of *Leuconostoc* dextransucrase (Bailey *et al.* 1957*a*) with the addition of D-arabinose. As with the *Leuconostoc* enzyme, fructose at high concentrations depresses the activity of *S. bovis* dextransucrase. Except for melibiose and arabinose all of the sugars which stimulate activity act as alternative glucosyl acceptors to produce oligosaccharides. Fructose, although it depresses activity, also gives rise to oligosaccharides; the same effect has been reported by Koepsell *et al.* (1953) for *Leuconostoc* dextransucrase. The main oligosaccharide produced by this enzyme in the presence of fructose and sucrose is a disaccharide leucrose (Stodola, Sharpe & Koepsell, 1956). Large amounts (representing up to 12% of the available sucrose) of a sugar apparently identical with leucrose have been observed in dextran-producing cultures of *S. bovis* strain I (Bailey & Oxford, 1958*a*).

All of the evidence available at present seems to indicate that the mechanism of dextran synthesis is the same for both *S. bovis* and *Leuconostoc* dextransucrase. The most interesting property of *S. bovis* dextransucrase is its occurrence in glucose cultures containing no added sucrose. No matter what explanation is offered for its presence in these cultures, they do provide a ready source of comparatively dextran-free dextransucrase.

SUMMARY

1. Dextransucrase has been isolated by ammonium sulphate precipitation from cell-free culture fluids of *Streptococcus bovis* grown on sucrose or glucose in the presence of carbon dioxide.

2. The dextransucrase isolated from the sucrose cultures contained 70% of polysaccharide and 1·79% of nitrogen, whereas the enzyme from the glucose cultures contained 4% of polysaccharide and 9·06% of nitrogen.

3. Both dextransucrase preparations had maximum activity in the range pH 5·0–6·5 and the temperature range 37–44°.

4. At pH 4·5 the enzyme was very unstable.

5. Maltose, isomaltose, isomaltotriose, glucose, galactose, methyl α-glucoside, lactose and cellobiose stimulated dextransucrase activity and acted as alternative glucosyl acceptors to produce oligosaccharides.

6. D-Arabinose and melibiose stimulated activity but did not act as glucosyl acceptors.

7. Fructose depressed dextransucrase activity but acted as an alternative glucosyl acceptor to produce oligosaccharides.

8. Dextransucrase preparations from both sucrose and glucose cultures gave good yields of dextran from sucrose.

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A Bacterial Dextranase

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Although several species of moulds, notably *Penicillium funiculosum* and *P. lilacinum* (Hulten & Nordström, 1949), have been shown to produce extracellular dextranases, members of only one bacterial genus (*Bacteroides*; Hehre & Sery, 1952) have been reported to produce this type of enzyme. In view of the possibility of dextran production by *Streptococcus bovis* in the rumen of cattle (Bailey & Oxford, 1958) we have been interested in the likelihood of rumen micro-organisms secreting dextranases. So far, two strains of an organism resembling *Lactobacillus bifidus* have been the only organisms possessing dextranase activity which one of us (R.T.J.C.) has isolated from the bovine rumen. As the end products of dextran hydrolysis by cell-free culture fluids of these organisms were rather unusual, it was decided to examine the properties of the dextranase. This paper reports the results obtained in a study of the dextranase preparations from cell-free culture fluids of these organisms and of one human strain of *L. bifidus*. For the purpose of this paper the two rumen organisms are referred to as strains of *L. bifidus*.

EXPERIMENTAL

Reducing sugars. Reducing sugars were measured in dextranase solutions, after neutralizing with 0·25 N-NaOH, by the cuprimetric method of Shaffer & Hartmann (1921). Preliminary experiments with isomaltodextrins showed that, with this copper reagent, a heating time of 15 min. was sufficient for full development of reducing power (cf. Turvey & Whelan, 1957). A standard curve was prepared with purified isomaltotriose, and all results were calculated in terms of this sugar. Although a mixture of oligosaccharides is produced by *L. bifidus* dextranase, only comparative reducing values were required and the triose standard was considered satisfactory.

Paper chromatographic analysis. Solutions were desalted with Dowex 50 and Dowex 2 ion-exchange resins. Chromatograms were developed with a solvent consisting of the top layer of ethyl acetate–water–pyridine (2:2:1, by vol.; Jermyn & Isherwood, 1949), and with a modified version of this solvent (Malpress & Hytten, 1958) which gave a better resolution of the higher oligosaccharides. Sugars were detected with silver nitrate (Trevelyan, Procter & Harrison, 1950) and aniline hydrogen phthalate (Partridge, 1949). Papers were also developed by the benzylamine–ninhydrin technique of Bayly & Bourne (1953).

Carbohydrates

Dextran. Dextran was isolated by ethanol precipitation (2 vol.) from a sucrose (4%, w/v) culture of *Streptococcus bovis* (strain I; Bailey & Oxford, 1958) which had been incubated for 48 hr. at 37° in an atmosphere of CO_2. The dextran was purified by the usual techniques and shown to be unbranched (Bailey, 1959). A standard solution of this dextran in water (10 mg./ml.) was prepared and, except where stated otherwise, used in all dextranase tests. In addition, a specimen of dextran produced from sucrose at 25° by *Betacoccus arabinosaceus* (*Leuconostoc mesenteroides*), Birmingham strain, was also used. It had been shown to consist of branched and unbranched chains (Bailey, unpublished work) and to contain 10–15% of branch (α-1:3-) linkages (Barker, Bourne, Bruce, Neely & Stacey, 1954).

Isomaltodextrins. S. bovis dextran (6 g.) was partially hydrolysed by heating at 100° in 0·33 N-H_2SO_4 (500 ml.) for 8 hr. After neutralizing ($BaCO_3$) and filtering, the hydrolysate was adsorbed on to a charcoal–Celite column (45 cm. × 3·5 cm.; Whistler & Durso, 1950). A batch of Darco charcoal (G60), which had been in store for several years and which had lost some of its absorption power, was used unwashed. After elution of glucose with water, ethanol–water mixtures eluted an apparently homologous series (by paper chromatography) of sugar fractions. The first two members of the series were chromatographically identical with authentic isomaltose and isomaltotriose (prepared from *Leuconostoc* dextran). As the *S. bovis* dextran was unbranched it was assumed that the other oligosaccharide fractions were members of the series isomaltotetraose–isomalto-octaose. Pure fractions were eluted with the following ethanol–water (v/v) mixtures:

isomaltose 1–2%, isomaltotriose 4–5%, isomaltotetraose 7–8%, isomaltopentaose 10–11%, isomaltohexaose 14–15%, isomaltoheptaose 20–21% and isomalto-octaose 26–28%. Each fraction was purified by the method of Whelan, Bailey & Roberts (1953) to yield 100–250 mg. of freeze-dried sugar. Paper-chromatographic analyses showed that each isomaltodextrin was free from its higher homologues and did not contain more than traces of its lower homologues.

Starch. Soluble starch (1 g.) was dissolved in water, dialysed at 2° against water and the solution diluted to contain 10 mg./ml.

Organisms

L. bifidus, D13/293, was isolated from the rumen contents of a fistulated cow fed on fresh red clover.

L. bifidus, D15/1, was isolated from the rumen contents of a second fistulated cow fed on hay.

L. bifidus, D17/H, was isolated from the faeces of a nursing infant.

The organisms were maintained by frequent subculture in a tomato-juice medium. The medium was a modification of that used by Rogosa, Wiseman, Mitchell, Disrealy & Beaman (1953) and contained: Bacto-Tryptose, 5 g.; Bacto-Tryptone, 5 g.; Bacto-Yeast Extract, 5 g.; Bacto-Beef Extract, 1 g.; glucose, 3 g.; lactose, 2 g.; Tween 80, 0·05 g. (Atlas Powder Co., N.Z.); canned tomato juice, 200 ml.; water to 1 l.; pH was 6·5–6·7.

Dextranase

Assay of dextranase activity. Dextranase activity may be measured by following either the changes in some physical property of the added dextran (e.g. opalescence or viscosity) or the production of reducing sugars from the dextran. The first method is used when the dextranase cleaves the dextran molecule at random (cf. α-amylase). The second method is preferable when the enzyme, from the start of hydrolysis, cleaves individual glucose or short-chain oligosaccharide units from the dextran molecule (cf. β-amylase). Preliminary experiments suggested that *L. bifidus* dextranase operated by the first type of mechanism. Dextranase activity of the enzyme preparations was therefore measured by following the loss of opalescence of dextran in standard solutions containing freeze-dried enzyme (5–20 mg./1 ml. of water) or cell-free culture fluid (1 ml.), dextran solution (2 ml.) and citrate buffer (0·2 M, pH 5·5; 2 ml.). A control solution containing dextran, buffer and boiled enzyme (15 min. at 100°) was included in each series of tests. The solutions were prepared in special nephelometer tubes and, unless stated otherwise, incubated at 37° under toluene. At the start, and at intervals during incubation, the opalescence of the enzyme solutions relative to the boiled enzyme control was measured in an EEL nephelometer (Evans Electroselenium Ltd., Harlow, Essex). Dextranase activity of a solution was reported as the percentage loss of opalescence of the solution after a stated time. Only comparative results were required in the present study and for this purpose the standard method proved satisfactory and reproducible.

Preparation of dextranase. For dextranase preparations the organisms were grown in a modification of the medium used by Hehre & Sery (1952), which contained: Bacto-Tryptose, 10 g.; Bacto-Yeast Extract, 0·3 g.; *S. bovis* dextran, 1·5 g.; tap water to 1 l.; pH was 7·2. Cultures were grown in an atmosphere of H_2+CO_2 (95:5, v/v) at 37° for 48 hr. Cell-free fluid was obtained by centrifuging cultures for 30 min. (30 000 *g*) on a Spinco preparative ultracentrifuge. The supernatant was recentrifuged to ensure complete removal of cells. Dextranase was precipitated by dissolving $(NH_4)_2SO_4$ (A.R.) in the cell-free fluid (60 g./100 ml.) and leaving the solution overnight at 2°. The precipitate was collected by centrifuging (30 000 *g*) at 0–5° for 30 min., dissolved in citrate buffer (0·05 M, pH 6·0; 50 ml.) and dialysed for 48 hr. at 2° against two changes (2 l.) of the citrate buffer in well-washed dialysis tubing. The final solution of enzyme in citrate buffer was centrifuged and freeze-dried to a light-brown powder which was stored at 2°.

RESULTS

Production of dextranase by Lactobacillus bifidus

The three strains of *L. bifidus* grew readily in the dextran medium; the cell-free culture fluids showed good dextranase activity within 48 hr. Free reducing sugars or oligosaccharides could not be detected in the growing cultures. The organism (strain D13/293) also grew readily in the same medium when the dextran was replaced by glucose (1%, w/v). However, the cell-free fluid from this culture had no dextranase activity.

Two freeze-dried dextranase preparations were obtained: dextranase I (0·35 g.; 5·7% N), isolated from 400 ml. of cell-free culture fluid of strain D13/293, and dextranase II (0·30 g.; 3·02% N), isolated from 200 ml. of cell-free culture fluid of strain D17/H. Suitable amounts of enzyme in the

Fig. 1. Rate of loss of opalescence of dextranase–dextran solutions. Solutions containing dextranase (in water, 1 ml.), dextran solution (2 ml.) and citrate buffer (0·2 M, pH 5·5; 2 ml.) were incubated at 37° and opalescence was measured at intervals. ●, Dextranase I (5 mg.); ○, dextranase II (20 mg.).

standard solutions were found to be dextranase I (5 mg.) and dextranase II (20 mg.) (see Fig. 1). For each enzyme preparation the rate of loss of opalescence in the standard solutions was proportional to the concentration of dextranase preparation within the range of dextranase I (2–10 mg.) and dextranase II (10–30 mg.) used. It was not possible to assess the absolute recovery of dextranase from cell-free culture fluids. Comparison of the dextranase activity of the final dialysed enzyme solution and of its freeze-dried solid suggested that there was little loss of activity on freeze-drying. Tests for dextranase activity of other strains of *L. bifidus* were made with cell-free culture fluids.

Action of dextranase on dextran

The action of dextranase on dextran was examined by incubating a standard solution containing dextranase I (5 mg.) and dextran and comparing this with control solutions containing (*a*) boiled enzyme and dextran, (*b*) dextran only and (*c*) dextranase only. After incubation for 2 hr. the opalescence due to added dextran had largely disappeared from the dextranase plus dextran solution but remained in the controls containing dextran. At this stage no reducing sugars could be detected on the paper chromatograms of any of the solutions. Incubation was continued for a further 16 hr. when paper chromatograms of the dextranase plus dextran solution, but not of the controls, showed the presence of an apparently homologous series of reducing-sugar spots. The members of the series were subsequently shown to be chromatographically identical with isomaltotriose, isomaltotetraose, isomaltopentaose, isomaltohexaose and longer-chain members of the series. The spots corresponding to the first three sugars were the most intense with the pentasaccharide spot the strongest of the three. Glucose and isomaltose were absent from the papers. Similar results were obtained with dextranase II and with the cell-free culture fluid of strain D15/1.

When dextranase I (5 mg.) was incubated with the *Leuconostoc* dextran the same rapid loss of opalescence occurred. Paper chromatograms of the solution, after incubation for 24 hr., showed a mixture of tri-, tetra- and penta-saccharides and higher saccharides. Glucose and isomaltose were again absent. It has not yet proved possible to establish whether the α-1:3-branch linkages of this dextran are attacked by the dextranase.

Strains of *L. bifidus* showed a marked fermentation of starch when grown in medium containing this carbohydrate. No sign of amylase activity could be detected in dextranase I (5 mg.) or dextranase II (20 mg.) when they were incubated with starch solution (2 ml.) under the standard conditions.

The loss of opalescence of dextran was followed in standard dextranase–dextran solutions containing either dextranase I (5 mg.) or dextranase II (20 mg.). The results obtained are shown in Fig. 1 and show a steady fall in opalescence during the first 2 hr. of incubation.

The rates of loss of opalescence and production of reducing sugars from dextran were compared in a dextranase (I)–dextran solution. At intervals during incubation opalescence was measured and portions (1 or 2 ml.) were withdrawn for the measurement of the reducing-sugar content (calculated as isomaltotriose). The results are shown in Fig. 2. It can be seen that the enzyme rapidly destroys opalescence but that reducing sugars are produced only after prolonged incubation.

Effect of pH *on dextranase activity*

The effect of pH on dextranase activity was measured in standard dextranase–dextran solutions (5 ml.) in which the pH of the added sodium citrate solution (0·2 M, 2 ml.) ranged from 4·0 to 7·3. The solutions, together with appropriate boiled enzyme controls, were incubated and the loss of opalescence was measured after incubation for 35 and 60 min. After the second measurement the pH of the solutions was determined. Under the conditions used pH had no effect on the opalescence of the dextran solution itself. The effect of ionic strength on dextranase activity was not examined.

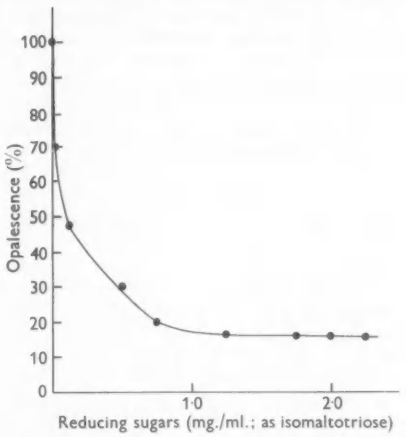

Fig. 2. Loss of opalescence and increase in reducing-sugar content of dextranase–dextran solution. Solution containing dextranase I (15 mg./3 ml. of water), dextran solution (6 ml.) and citrate buffer (0·2 M, pH 5·5; 6 ml.) was incubated for 30 hr. at 37°. Opalescence and reducing-sugar content (in 1 ml.) were measured at intervals.

Such effects have not been reported for mould dextranase (Tsuchiya, Jeanes, Bricker & Wilham, 1952) or *Bacteroides* dextranase (Sery & Hehre, 1956). The results obtained with dextranase I (5 mg.) are shown in Fig. 3. Similar results were obtained with dextranase II (20 mg.).

From a study of the effect of pH on the activity of *Bacteroides* dextranase, Sery & Hehre (1956) concluded that two enzymes were present. One enzyme caused a rapid and profound change in the physical properties of dextran and had optimum activity over the range pH 4·8–5·4 but negligible activity above pH 6·5. The second enzyme liberated glucose by endwise cleavage of the dextran molecule with little immediate effect on its physical properties. This enzyme had optimum activity over the range pH 7·0–7·2 and practically no activity below pH 6·0. This latter enzyme was detected by examining the effect of pH on the rate of release of reducing sugars from the dextran.

The possible presence of a second dextranase in dextranase I was tested for by incubating a series of standard dextranase–dextran solutions, at the various pH values, for 8 hr. and measuring the reducing-sugar content (as isomaltotriose) of each solution. The results showed that maximum release of reducing sugars occurred in the range pH 5·5–6·5. This was the pH range in which maximum loss of opalescence occurred (see Fig. 3). The dextranase–dextran solution of pH 7·3 was incubated for a further 16 hr. and examined by paper chromatography. The same mixture of oligosaccharides as was detected in the standard dextranase–dextran solution (pH 5·5) was present on the papers at a lower concentration.

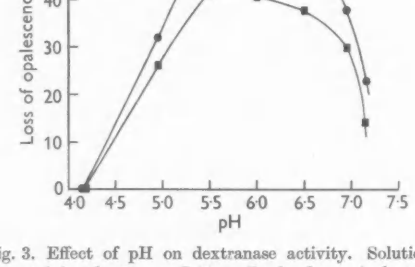

Fig. 3. Effect of pH on dextranase activity. Solutions containing dextranase I (5 mg./1 ml. of water), dextran solution (2 ml.) and citrate solution (0·2 M, 2 ml.) were incubated at 37°. ■, Loss of opalescence after incubation for 35 min.; ●, loss of opalescence after incubation for 60 min.

Effect of temperature on dextranase activity

The effect of temperature on dextranase activity was measured in standard solutions of dextranase I (5 mg.) and dextran. The solutions, and boiled enzyme controls, were incubated at various temperatures and the loss of opalescence was measured after 30 min. The results obtained are shown in Fig. 4. Although the enzyme had maximum activity in the temperature range 41–50°, it was more convenient to use a temperature of 37° for the investigations of dextranase activity.

Action of dextranase on isomaltodextrins

The action of dextranase on members of the isomaltodextrin series of oligosaccharides was tested by incubating standard solutions containing dextranase I (5 mg.) and isomaltodextrin (20 mg./2 ml. of water) instead of dextran. Control solutions containing (*a*) isomaltodextrin but no enzyme and (*b*) dextranase only were also incubated. At the commencement and after incubation for 4 and 24 hr., portions (1 ml.) of each solution were withdrawn for measurement of reducing sugars. Results obtained, expressed as the percentage increase in reducing power relative to the initial values, are listed in Table 1.

Before incubation and after incubation for 24 hr. the solutions were analysed for sugars by

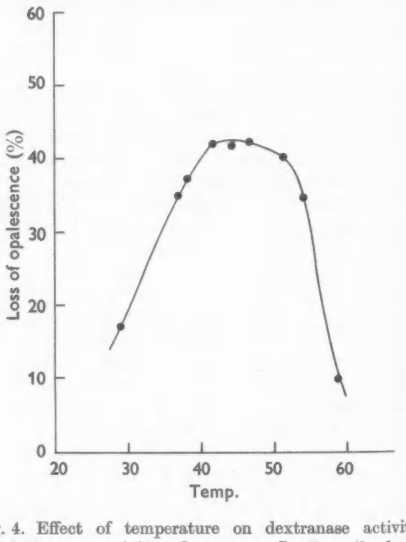

Fig. 4. Effect of temperature on dextranase activity. Solutions containing dextranase I (5 mg./1 ml. of water), dextran solution (2 ml.) and citrate buffer (0·2 M, pH 5·5; 2 ml.) were incubated for 30 min. and loss of opalescence was measured.

Table 1. *Action of dextranase on isomaltodextrins*

Solutions containing dextranase I (5 mg./1 ml. of water), isomaltodextrin (20 mg./2 ml. of water) and citrate buffer (0·2 M, pH 5·5; 6 ml.) were incubated at 37°. Reducing-sugar content was measured at intervals. The intensity of the sugar spots is indicated by the grading system: + (trace) to + + + + (intense).

Isomaltodextrin	Percentage increase in reducing sugar value after incubation for 4 hr.	Percentage increase in reducing sugar value after incubation for 24 hr.	Additional oligosaccharides detected after incubation for 24 hr.
Isomaltose	0	0	None
Isomaltotriose	0	0	None
Isomaltotetraose	0	5·0	None
Isomaltopentaose	1·4	2·4	None
Isomaltohexaose	4·2	12·5	Trisaccharide (+)
Isomaltoheptaose	19·4	50·6	Tri- and tetra-saccharide (both + +)
Isomalto-octaose	37·0	83·0	Tri- and penta-saccharide (both + + + +); tetra-saccharide (+ +)

paper chromatography. Oligosaccharides, other than added isomaltodextrins, which were detected on chromatograms of the dextranase–isomaltodextrin solutions after incubation for 24 hr., are listed in Table 1. Only the added isomaltodextrins were detected in the initial samples, the control solutions and the enzyme solutions, which gave no increase in reducing power. Comparison of the chromatograms of the solutions initially and after incubation for 24 hr. indicated no loss of added isomaltodextrin after incubation except in the dextranase solutions containing these isomaltodextrins: isomaltohexaose (slight loss), isomaltoheptaose (about 50% loss), isomalto-octaose (almost disappeared).

Growth of the organism on isomaltodextrins

As the organisms grew profusely with dextran as the major carbon source, it was presumed that they would utilize the products of dextranase action on dextran. This was confirmed by growing *L. bifidus* (strain D13/293) in a medium with a mixture of equal amounts of isomaltotetraose and isomaltopentaose as carbon source. Good growth was obtained with disappearance of the isomaltodextrins, as shown by paper chromatography.

DISCUSSION

Moulds are the commonest source of extracellular dextranase. With the exception of a strain of *Aspergillus niger*, which produces glucose only (Tsuchiya *et al.* 1952), the products of the attack of mould dextranases on dextran are isomaltose and smaller amounts of glucose and isomaltotriose (Tsuchiya *et al.* 1952; Jeanes, Wilham, Jones, Tsuchiya & Rist, 1953). The mould dextranases appear to act by random cleavage of the dextran molecule. A pronounced change in the physical properties of the dextran occurs before free sugar (isomaltose or glucose) is liberated (Hulten & Nordström, 1949; Sery & Hehre, 1956). They attack some but not all highly branched dextrans at the same rate as unbranched dextrans. The dextranase of intestinal species of the bacterial genus *Bacteroides*, unlike most of the mould enzymes, converts dextran into glucose only (Hehre & Sery, 1952; Sery & Hehre, 1956). However, this dextranase consists of a mixture of two enzymes, one which cleaves the dextran molecule at random and one which carries out an endwise cleavage of glucose from the dextran chain (Sery & Hehre, 1956). Attack on branched dextrans by this latter enzyme appears to be limited.

Lactobacillus bifidus appears to be only the second bacterial species which has been shown to produce a dextranase. Tsuchiya *et al.* (1952) tested 200 species of bacteria and yeasts for dextranase activity but obtained only negative results. From the study of the effect of pH on dextranase activity it appears that the isolated enzyme, unlike *Bacteroides* dextranase, contains only one dextranase with a pH optimum between 5·4 and 6·5. It is evident also that this enzyme hydrolyses dextran by a random cleavage of the molecule. An initial profound change in the physical properties (e.g. opalescence) of the dextran was followed, much later, by the appearance of reducing sugars (Figs. 1 and 2). The enzyme attacked both unbranched *S. bovis* dextran and a branched *Leuconostoc* dextran.

The mixture of oligosaccharides produced is the most unusual feature of the action of *L. bifidus*

dextranase on dextran. Glucose and isomaltose, the main products of the action of other dextranases on dextran, are not produced. The accumulation of tri-, tetra-, penta- and higher oligo-saccharides suggests that the shortest isomaltodextrin chain which the enzyme can attack is one composed of six glucose units. Although the action of the dextranase on individual isomaltodextrins is in agreement with the accumulation of tri-, tetra-, penta- and hexa-saccharides, it indicates that the shortest chain readily cleaved by the enzyme is in fact one composed of seven glucose units. The need for a substrate of such long chain length is of interest. All other dextranases are capable of hydrolysing chains of from two to four glucose units in length, as are most α-amylases and cellulases. Members of the oligosaccharide mixture which result from dextranase activity are capable of supporting the growth of *L. bifidus*.

Although three strains of *L. bifidus* which secrete dextranase have been obtained, two other human strains of the organism which did not produce dextranase have been isolated. Strains of *Lactobacillus brevis*, *L. bulgaricus*, *L. acidophilus* and *L. plantarum* (one of each) were also found to be without action on dextran. A more detailed examination of the distribution of this unusual dextranase in strains of lactobacilli appears to be warranted.

SUMMARY

1. Two bovine rumen strains and one human strain of *Lactobacillus bifidus* have been shown to secrete an extracellular dextranase.

2. The dextranase was isolated from cell-free culture fluids by precipitation with ammonium sulphate.

3. Although the enzyme rapidly destroyed the opalescence of a dextran solution, reducing sugars were produced only after prolonged incubation.

4. The products of the action of the enzyme on dextran were a mixture of isomalto-triose, -tetraose, -pentaose and higher oligosaccharides. Glucose and isomaltose were not produced.

5. Isomaltose, isomalto-triose, -tetraose and -pentaose were not attacked by the enzyme. Isomaltohexaose was only slightly hydrolysed, whereas isomalto-heptaose and -octaose were hydrolysed readily.

6. The dextranase had maximum activity in the range pH 5·4–6·5 and the temperature range 40–50°.

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The Initiation of Deoxyribonucleic Acid Synthesis in the Connective-Tissue Cell, with some Observations on the Function of the Nucleolus

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The zone of cells surrounding the explant in a conventional tissue culture enlarges by the emigration of cells from the explanted tissue and by the multiplication of cells which have emigrated. It is not clear, however, whether cells multiply within the explant before emigration or whether emigration necessarily precedes multiplication. The experiments described in the present paper had three aims: to determine when the synthesis of deoxyribonucleic acid in the cells of the emigration zone began; to measure the duration of the phase of synthesis of deoxyribonucleic acid in the individual cell and to establish the relation of this phase to the time of mitosis; and to examine the connexion between the synthesis of deoxyribonucleic acid and the synthesis of protein and ribonucleic acid. The

work involved identifying and counting those cells in the emigration zone which, at a given time, were synthesizing deoxyribonucleic acid. This was done by preparing radioautographs of the emigration zone after the cells had been exposed for a suitable period to tritium-labelled thymidine, it being generally accepted that this compound is incorporated into deoxyribonucleic acid only. The high resolution of radioautographs produced by tritiated thymidine is well known. In the present experiments the resolution was even further enhanced by the fact that connective-tissue cells, when grown on a glass surface, spread out and become very flat. When fixed they rarely exceed 1 μ in thickness. The radioautographs obtained from these cells were precise enough to permit certain observations to be made on the nucleoli. These observations indicate the site at which the synthesis of deoxyribonucleic acid is initiated in the cell.

EXPERIMENTAL

The connective-tissue cells were derived from the hearts of 5-day-old rats. Small fragments were cut from the heart and explanted on to the surface of microscope slides. About 50 fragments were deposited on each slide under a thin film of a medium consisting of 20% of bovine serum and 80% of Eagle's solution (Eagle, Oyama, Levy, Horton & Fleischman, 1956). The slides were kept for about 1 hr. at room temperature in moist chambers, by which time the fragments had stuck to the glass. No plasma was used to anchor the explants. The cells therefore emigrated from the tissue directly on to the glass surface and the resulting emigration zones consisted of a single layer of flattened cells. The slides were incubated at 37° in glass flasks containing enough of the same medium to cover the explants completely.

In order to determine which of the cells in the emigration zone were synthesizing deoxyribonucleic acid (DNA) the preparations were incubated for a given time with tritiated thymidine (supplied by Schwarz Laboratories Inc., N.Y., U.S.A.) at a concentration of 0·5 μc/ml. of medium. The slides were then removed from the flasks, washed four times in 0·85% NaCl soln. and then fixed by immersion for 15 min. in methanol. After fixation the slides were tapped gently so that the fragments of explanted tissue were dislodged; the cells of the emigration zones, however, remained adherent to the slide. These cells were extracted with ice-cold 5% (w/v) trichloroacetic acid and washed under running water for an hour or two. Stripping film (Kodak AR 10) was then laid down over the slides and developed after 7 days. Finally the cells were stained through the film with Nuclear-fast red.

In the experiments in which the duration of the phase of DNA synthesis was determined, it was necessary to permit the cells to continue growing after a given period of exposure to the labelled thymidine. In these cases the slides were removed from the flasks after the period of incubation with labelled thymidine, washed several times in 0·85% NaCl soln. containing an excess of unlabelled thymidine and then transferred to other flasks containing thymidine-free medium. In other experiments, which are described later, the cells of the emigration zones were brought into suspension by treatment with trypsin and then cultivated in small coverslip chambers. (For details see Harris, 1955*a*, *b*, 1956.)

In order to determine which of the cells were synthesizing ribonucleic acid (RNA) at any given time the preparations were incubated for 1 hr. in medium containing [8-^{14}C]-adenine (supplied by The Radiochemical Centre, Amersham, Bucks) at a concentration of 2 μc/ml. and then fixed. The cells were extracted with ice-cold 5% (w/v) trichloroacetic acid and washed under running water. Some of the preparations were then incubated for 2 hr. at 37° with crystalline pancreatic ribonuclease at a concentration of 0·5 mg./ml. in phosphate buffer, pH 6·7. Radioautographs of the preparations which had been treated with ribonuclease were compared with radioautographs of those which had not. Labelled adenine which was not extracted by ice-cold 5% (w/v) trichloroacetic acid but which was extracted by ribonuclease was considered to have been incorporated into RNA.

To determine which of the cells were synthesizing protein the preparations were incubated for 1 hr. in medium containing [1-^{14}C]DL-valine (supplied by The Radiochemical Centre) at a concentration of 2 μc/ml. and then fixed. The cells were extracted for 30 min. with 5% (w/v) trichloroacetic acid at 90° and for a further 30 min. with hot ethanol–ether (1:1). Radioautographs were then prepared. Labelled valine, which was not extracted by the above procedures, was considered to have been incorporated into protein.

RESULTS AND DISCUSSION

Initiation of synthesis of deoxyribonucleic acid

Connective-tissue cells made their appearance at the edges of the explants after incubation for about 8 hr. At this time and at intervals afterwards each of a series of fourteen preparations was exposed for 1 hr. to tritiated thymidine and then fixed. Radioautographs were prepared and the proportion of cells synthesizing DNA was determined for each slide. The results are illustrated in Fig. 1. It will be seen that only 10–15% of the earliest cells to appear were synthesizing DNA at the time of their emigration from the explant. Between the eighth and twentieth hours of incubation there was no increase in the proportion of cells synthesizing DNA, although by the end of this time the emigration zones contained several hundred cells. After 20 hr. the percentage of cells synthesizing DNA began to rise, reaching a maximum of 50–60% between 40 and 48 hr. Thereafter the percentage of cells synthesizing DNA fell gradually.

The above findings do not exclude the possibility that the cells which were not synthesizing DNA at the time of emigration from the explant might have synthesized DNA in the 8 hr. or so preceding emigration. This possibility was eliminated by the following experiment. The explants were put into medium containing tritiated thymidine from the outset and incubated in this medium until a small

number of cells had emigrated (a period of about 10 hr.). The percentage of labelled cells in the emigration zones was then determined. Again, only 10–15 % of the cells were found to be labelled; the great majority had thus not synthesized any DNA before emigrating. It is possible that even in 10 hr. the labelled thymidine might not have penetrated to the centre of the explants; but there is no reason to suppose that it would not have reached the cells at the edges of the explants, and these are the first to emigrate. It can be concluded then that 85–90 % of the cells emigrate before they begin to synthesize DNA and that there is a lag period of about 20 hr., of which the cells spend 12 hr. outside of the explant, before DNA synthesis begins.

The small proportion of cells which synthesize DNA either before or during emigration are presumably cells which were in the phase of DNA synthesis, or about to enter this phase, when the animal was killed. The heart of a 5-day-old rat is a rapidly growing structure, and one would therefore expect that at any one time a certain proportion of the cells in it would be in the process of synthesizing DNA.

Duration of the phase of synthesis of deoxyribonucleic acid

To determine the duration of the phase of DNA synthesis and its relationship in time to mitosis the following experiment was carried out. Twenty-four slides, each with about 50 explants attached, were incubated in non-radioactive medium for 30 hr., by which time emigration zones containing several hundred cells had been formed around most of the explants. At successive hourly intervals over the next 24 hr. tritiated thymidine was added to each of the flasks in turn. Each slide was incubated in the medium containing the tritiated thymidine for 1 hr., then washed in unlabelled thymidine and transferred to thymidine-free medium. At the end of 24 hr. all the slides were fixed and radioautographs prepared. All the cells in metaphase, anaphase or telophase in each slide (between 50 and 100) were examined and the proportion showing labelled chromosomes determined. The passage from metaphase to telophase takes no more than a few minutes in these cells. By selecting such mitotic figures in the emigration zones for examination groups of cells were obtained which were synchronized in these stages of mitosis at the time of fixation; and each of these cells had been exposed to tritiated thymidine at a known period before mitosis. If the chromosomes in a mitotic figure were labelled then that cell had obviously been in the phase of DNA synthesis at the time of exposure to the labelled thymidine; if the chromosomes were not labelled the cell was presumed not to have been in this phase at the time of exposure. From the proportion of labelled mitotic figures in each of these slides an estimate of the duration and timing of the phase of DNA synthesis was made.

The results of two such experiments are superimposed on one graph in Fig. 2. There is great variation in the duration and time of onset of DNA synthesis in different cells and less variation in the time at which DNA synthesis ceases. A median value derived from several hundred cells puts the beginning of DNA synthesis between 13 and 14 hr.

Fig. 1. Initiation of DNA synthesis in the cells of the emigration zone. The cells first make their appearance at the edges of the explants after incubation for 8 hr. Between 85 and 90% of them are not synthesizing DNA at this stage; they begin to do so after a lag period of about 20 hr. Each point represents a count of all the cells in six emigration zones.

Fig. 2. Duration of the phase of DNA synthesis and its relation to the time of mitosis. There is wide variation in the time at which the phase of DNA synthesis begins, but less variation in the time at which it ends. A period of at least an hour, but generally longer, elapses between the end of DNA synthesis and mitosis. M, Mitosis; ○, expt. 1; ●, expt. 2. Each point represents between 50 and 100 mitotic figures.

before metaphase and the end of DNA synthesis between 3 and 4 hr. before metaphase. Despite the variation from cell to cell it is evident that the phase of DNA synthesis occurs in the latter part of the intermitotic period and that there is an interval of at least an hour, but usually longer, between the end of DNA synthesis and metaphase. These findings are in general agreement with those obtained by Firket & Verly (1958) with chick-embryo connective-tissue cells.

Effect of inhibiting synthesis of normal protein and ribonucleic acid in the lag phase

With the incorporation of [1-^{14}C]valine and [8-^{14}C]adenine, under the conditions described, as criteria it was shown by radioautography that all the cells synthesized protein and RNA continuously. It was of interest to determine whether synthesis of protein and RNA was a prerequisite of DNA synthesis. The amino acid analogues DL-β-2-thienylalanine, DL-*p*-fluorophenylalanine, 3-fluoro-DL-tyrosine and DL-canavanine were used to interfere with the synthesis of protein, and 8-azaguanine and 5:6-dichloro-1-(β-D-ribofuranosyl)-benzimidazole with the synthesis of ribonucleic acid.

Each of the amino acid analogues had previously been found to inhibit the multiplication of the connective-tissue cells (H. Harris, unpublished work). Thienylalanine at a concentration of 5 mM, fluorophenylalanine at 2 mM, fluorotyrosine at 2 mM and canavanine at 10 mM arrested multiplication of the cells without killing them within 24 hr. If cells in which multiplication had been arrested by the analogue were transferred to medium free of the analogue multiplication resumed after a lag of 30–40 hr. The effects of thienylalanine and fluorophenylalanine could be reversed by phenylalanine, the effect of fluorotyrosine by tyrosine and that of canavanine by arginine. With thienylalanine, alkaline hydrolysis of the cell protein showed that the analogue had actually been incorporated. Similar analyses were not carried out with the other analogues used, although it seems likely from work with bacteria (Munier & Cohen, 1956) that these analogues are also incorporated into the protein.

Twelve slides, each with about 50 explants, were incubated in the usual medium for 18 hr. Two slides were exposed to labelled thymidine for 1 hr. at this stage and then fixed; radioautographs of these showed that 85–90% of the cells in the emigration zones were still in the lag phase. The remaining slides were washed several times in 0·85% NaCl soln. and divided into two groups. One group was transferred to flasks containing the usual medium, the other to flasks containing medium free from phenylalanine but with thienylalanine at a concentration of 5 mM. Incubation was continued. Every 2 hr. one slide from each group was exposed to labelled thymidine for 1 hr. and then fixed; the percentage of cells synthesizing DNA was determined for each preparation. The results of the experiment are shown in Fig. 3. It will be seen that in the control group the percentage of cells synthesizing DNA rises as usual after the twentieth hour of incubation. In the preparations exposed to thienylalanine this rise is abolished. It thus appears that when normal protein synthesis in the lag phase is prevented by the use of thienylalanine synthesis of DNA does not occur. But the synthesis of DNA is not immediately interrupted by transfer of the cells to the medium containing thienylalanine. The small proportion of cells in which DNA synthesis had already begun at the time of the transfer continued to synthesize DNA for some time; in a few cells synthesis of DNA could still be detected 8 hr. after the transfer. Grain counts showed, however, that although these cells continued to synthesize DNA in the presence of thienylalanine the rate of synthesis fell progressively. As judged by grain counts the amount of labelled thymidine incorporated by DNA-synthesizing cells between the seventh and eighth hour after their transfer to

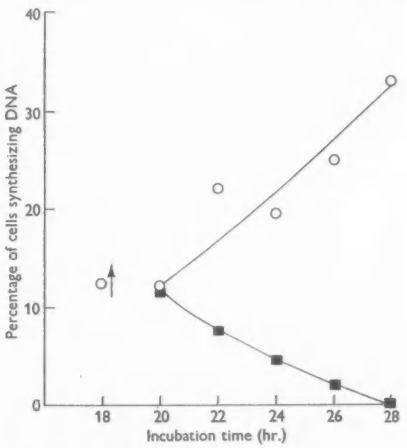


Fig. 3. Effect of DL-β-2-thienylalanine on the initiation of DNA synthesis. The rise in the percentage of cells synthesizing DNA which ordinarily follows the lag phase is abolished, but cells already synthesizing DNA continue to do so for some hours. Preparations were transferred to fresh media at the point indicated by the arrow. ○, Controls; ■, medium contains DL-β-2-thienylalanine at a concentration of 5 mM, but no phenylalanine. Each point represents a count of all the cells in six emigration zones.

medium containing thienylalanine was less than 25% of the amount incorporated in the corresponding period by the controls.

Essentially the same results were obtained with fluorophenylalanine at a concentration of 2 mM, fluorotyrosine at 2 mM and canavanine at 10 mM in media from which the corresponding natural amino acid had been omitted.

The effects produced by 8-azaguanine and the dichlorobenzimidazole riboside were less clear-cut than those produced by the amino acid analogues. This was perhaps because neither of these compounds, nor any other, suppresses the synthesis of natural RNA completely. Azaguanine forms spurious RNA, but apparently permits some normal RNA synthesis to continue (Mandel & Markham, 1958); and even at concentrations approaching the limit of solubility the dichlorobenzimidazole riboside does not abolish the incorporation of labelled adenosine into RNA completely (Tamm, 1958).

8-Azaguanine, at a concentration of 0·01 mM, arrested the multiplication of the connective-tissue cells without killing them within 24 hr. The effect produced at a concentration of 0·1 mM appeared to be similar, but at 1 mM some cells died within 24 hr. The dichlorobenzimidazole riboside at the limit of solubility arrested cell multiplication, but also caused the death of some cells within 24 hr. At one-tenth of this concentration, cell multiplication was not arrested.

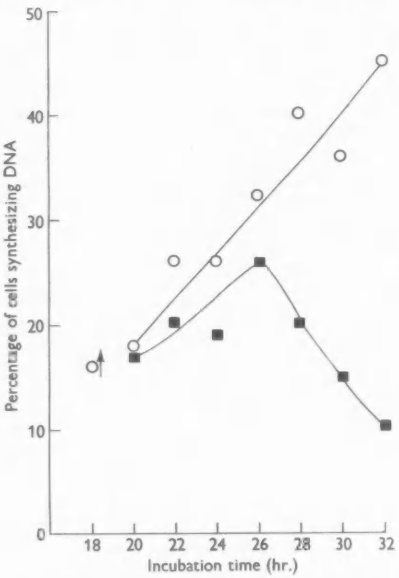


Fig. 4. Effect of 8-azaguanine on the initiation of DNA synthesis. The rise in the percentage of cells synthesizing DNA which ordinarily follows the lag phase is not abolished, but it is more gradual and reaches a much lower level than in the controls. Preparations were transferred to fresh medium at the point indicated by the arrow. ○, Controls; ■, medium contains 8-azaguanine at a concentration of 0·5 mM. Each point represents a count of all the cells in six emigration zones.

The effect of exposing cells in the lag phase to 8-azaguanine is shown in Fig. 4. The preparations were incubated for 18 hr. in the usual medium and then transferred to medium containing 8-azaguanine at a concentration of 0·5 mM. It will be seen that the rise in the percentage of cells synthesizing DNA which ordinarily occurs at the end of the lag phase was not completely abolished, but it was perhaps more gradual than in the controls and it reached a much lower level. Thus although azaguanine introduced in the lag phase did not prevent the onset of DNA synthesis in all cells, it did prevent it in most. Like the amino acid analogues, however, azaguanine did not immediately interrupt the synthesis of DNA once this had begun. Cells which were in the phase of DNA synthesis at the time of transfer to medium containing azaguanine continued to synthesize DNA for many hours, although grain counts showed a progressive fall in the rate of DNA synthesis.

The dichlorobenzimidazole riboside at the limit of solubility gave similar results.

Although the experiments with azaguanine and the dichlorobenzimidazole riboside are less convincing than those with the amino acid analogues, they indicate none the less that interference with normal RNA synthesis in the lag phase prevents the onset of DNA synthesis in most cells. If normal RNA synthesis could be completely suppressed or vitiated without killing the cells, it is possible that the results would more closely resemble those obtained with the amino acid analogues.

EXPLANATION OF PLATE 1

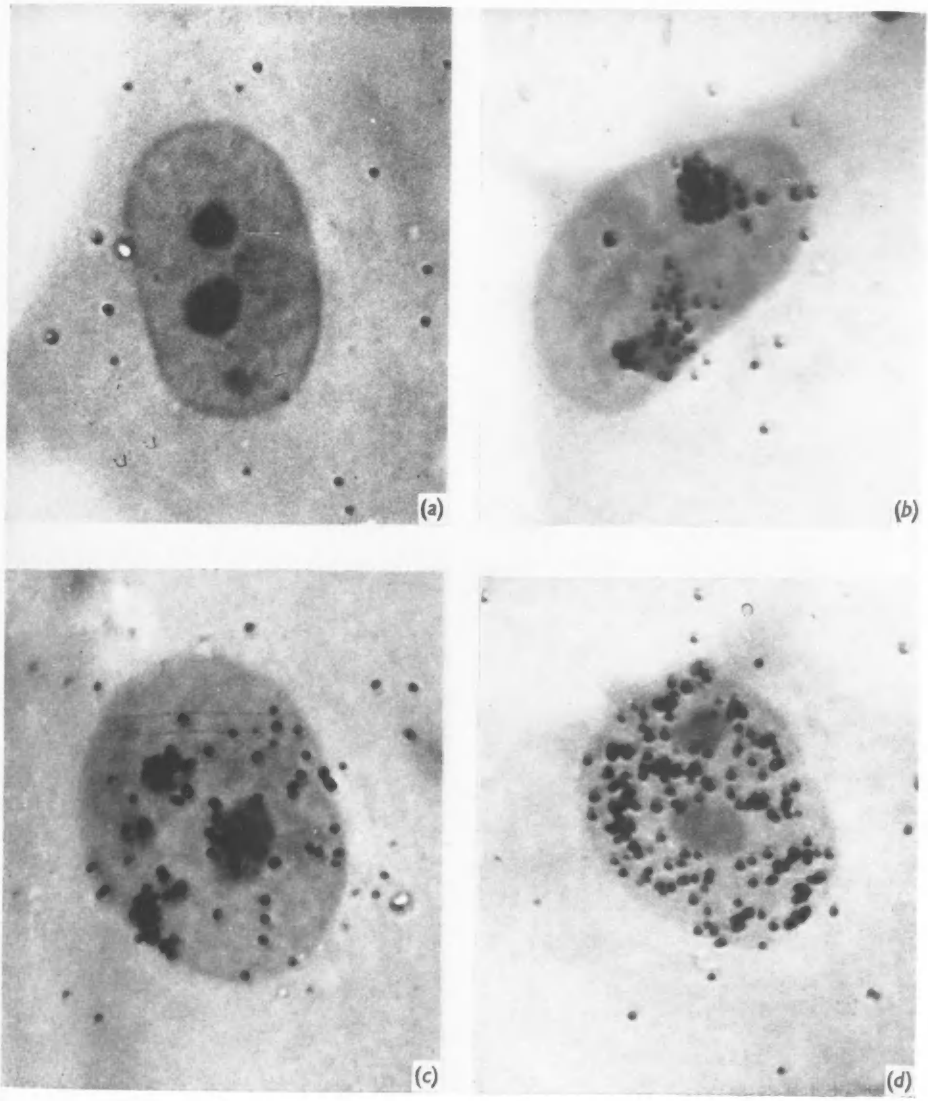
Radioautographs of the nuclei of connective-tissue cells cultivated in medium containing tritiated thymidine. The cells were stained through the film with Nuclear-fast red. Magnification × 2600.

(*a*) The nucleus shows two prominent nucleoli and one smaller one. There is no labelling over any part of the nucleus.

(*b*) The labelling is over the nucleoli or in close association with them; the rest of the nucleus has no more activity than the background.

(*c*) There is quite heavy labelling over the nucleoli and some labelling over the rest of the nucleus.

(*d*) There is no labelling over the nucleoli whereas the rest of the nucleus is heavily labelled.



H. HARRIS—THE INITIATION OF DEOXYRIBONUCLEIC ACID SYNTHESIS IN THE CONNECTIVE-TISSUE CELL, WITH SOME OBSERVATIONS ON THE FUNCTION OF THE NUCLEOLUS

(*Facing p.* 58)

Role of the nucleolus in the initiation of synthesis of deoxyribonucleic acid

The nucleoli in the connective-tissue cell are very prominent structures (Plate 1 *a*). There are usually one, two or three nucleoli per cell, but more than three are occasionally found. During the course of the present experiments it was noticed that the grains in radioautographs of cells which had been exposed to tritiated thymidine were not randomly distributed over the nucleus. Three patterns of labelling emerged. They are shown in Plate 1 (*b*, *c*, *d*). In cells which had been exposed to the labelled thymidine for an hour these patterns were often obscured by the density of the radioautographs; but if the time of incubation with tritiated thymidine was reduced to 5 or 10 min. the three types of labelling became very clear. In Plate 1 *b* the labelling is over the nucleoli or in close association with them; the rest of the nucleus has no more activity than the background. In Plate 1 *c* there is quite heavy labelling over the nucleoli and some labelling over the rest of the nucleus. When both the nucleolus and the rest of the nucleus are labelled the relative intensity of labelling may vary: either one may be more heavily labelled than the other. In Plate 1 *d* there is no labelling over the nucleoli whereas the rest of the nucleus is heavily labelled.

Some indication of the duration of the three phases represented by these three types of radioautograph could be obtained from their relative frequencies in a population of cells multiplying at random. Although strict randomness is difficult to achieve, satisfactory conditions were obtained in populations of dissociated connective-tissue cells grown from standard inocula in coverslip chambers. The technique has been previously described (Harris, 1955*a*). The multiplication of cells grown in this way shows no detectable synchronization after the first 48 hr. Incubation of such cells with tritiated thymidine for 5 min. periods gave radioautographs in which about 55% of the labelled cells showed no labelling over the nucleoli, about 40% labelling over nucleoli and the rest of the nucleus and 5% or less labelling over the nucleoli only. It can be inferred then that in a phase of DNA synthesis lasting 10 hr. the stage represented by Plate 1 *b* probably lasts half an hour or less, that represented by Plate 1 *c* about 4 hr. and that represented by Plate 1 *d* 5–6 hr.

Unless either nucleolar labelling or labelling over the rest of the nucleus is initiated, suspended for a period of time and then resumed, which seems very unlikely, the stage represented by Plate 1 *c* must be intermediate in time between that represented by Plate 1 *b* and that represented by Plate 1 *d*. But none of the data so far presented permits one to decide whether the stages showing nucleolar labelling precede or succeed the stage showing no nucleolar labelling. This problem was resolved by studying a population of cells in which some measure of synchronization had been produced. If most of the cells in a population are synchronized in the phase preceding DNA synthesis and then permitted to pass into and out of the phase of DNA synthesis, the incidence of any event occurring in the early part of the phase of DNA synthesis will rise as the cells pass into this phase and fall as the cells pass out of it; conversely, the incidence of any event occurring in the latter part of the phase of DNA synthesis will fall as the cells pass into this phase and rise as the cells pass out of it. Such conditions are to be found in the emigration zone when the cells pass from the lag phase into the phase of DNA synthesis, but more marked synchronization can be produced when the cells of the emigration zone are brought into suspension by treatment with trypsin and then cultivated as

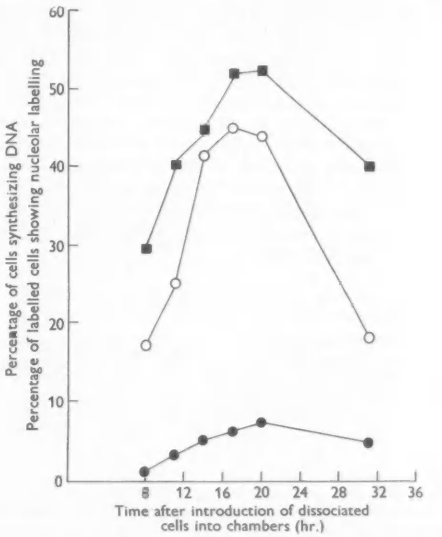


Fig. 5. Variation in the proportion of labelled cells showing nucleolar labelling during the period when most of the cells in a partially synchronized population pass into and out of the phase of DNA synthesis. As increasing numbers of cells pass into the phase of DNA synthesis, the percentage of labelled cells showing nucleolar labelling rises; as increasing numbers pass out of this phase the percentage of labelled cells showing nucleolar labelling falls. ○, Percentage of cells synthesizing DNA; ■, percentage of labelled cells showing either form of nucleolar labelling (Plate 1 *b* or *c*); ●, percentage showing labelling in association with the nucleolus only (Plate 1 *b*). Each point represents a count of 600 cells.

dissociated cells (Harris, 1955*a*). It was found that after the cells had been dissociated and had again become adherent to the glass surface on which they were to be grown, only 10 % of them or less were in the phase of DNA synthesis. During the next 36 hr. the percentage of cells synthesizing DNA rose rapidly and later fell rapidly. The fluctuations in the proportion of labelled cells showing nucleolar labelling during this period are set out in Fig. 5. The cells were exposed to tritiated thymidine for 5 min. periods at the times shown. It will be seen that as increasing numbers of cells move into the phase of DNA synthesis the percentage of labelled cells showing nucleolar labelling rises; and as increasing numbers move out of the phase of DNA synthesis the percentage showing nucleolar labelling falls. This is the case both for the stage represented by Plate 1*b* and that represented by Plate 1*c*. Conversely, the percentage of labelled cells showing no nucleolar labelling (Plate 1*d*) falls as increasing numbers of cells move into the phase of DNA synthesis and rises as increasing numbers move out of it. It follows then that the stages showing nucleolar labelling precede the stage showing no nucleolar labelling and that the probable sequence of events is represented by Plate 1*b*, *c* and *d*, in that order. There are therefore good grounds for concluding that the process of DNA synthesis is initiated at the nucleolus. Too little is known about the nucleolus to permit useful speculation at this stage about the possible biochemical role which it might play in this connexion; this problem is under current investigation.

SUMMARY

1. The observations described in the present paper were made on connective-tissue cells derived from explants of rat-heart tissue.

2. Between 85 and 90 % of these cells emigrate from the explants before they begin to synthesize deoxyribonucleic acid. The lag period preceding the onset of synthesis of deoxyribonucleic acid lasts about 20 hr.; for 12 hr. of this phase, the cells are outside the explant.

3. During the lag period there is continuous synthesis of both protein and ribonucleic acid. These two processes are prerequisites for the synthesis of deoxyribonucleic acid: if either is interfered with this latter synthesis does not take place.

4. The duration of the phase of synthesis of deoxyribonucleic acid varies widely. A median figure, obtained from several hundred cells, puts the beginning of this phase between 13 and 14 hr. before metophase and its end between 3 and 4 hr. before metaphase; mitosis is thus immediately preceded by a period in which no synthesis of deoxyribonucleic acid occurs.

5. An analysis of the patterns of nuclear and nucleolar labelling in radioautographs of cells exposed to tritiated thymidine indicates that the synthesis of deoxyribonucleic acid is initiated at the nucleolus.

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A Substance from Animal Tissues which Induces Ketonuria

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Before insulin was generally available Petrén (1924) found that the addition of meat to the diet of diabetic patients could exacerbate or induce ketosis, whereas a diet containing a high proportion of fat reduced ketosis. Marks & Young (1939) made similar observations with dogs in which metahypophyseal diabetes had been induced. A reduction of ketonuria has also been observed when a high-fat diet is fed to alloxan-diabetic rats (Burn, Lewis & Kelsey, 1944; Bornstein & Nelson, 1949), and the exacerbating effect of dietary meat on the ketosis of alloxan-diabetic rats has been observed by Lazaris & Brzezhinskaya (1946).

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Marks & Young (1939) found also that if the meat in the diet of a diabetic dog was replaced by an amount of casein equivalent on the basis of total nitrogen, an existing ketonuria was greatly reduced. Moreover if, to a diet rich in fat, an aqueous extract of meat was added, ketonuria was greatly increased, though the residue left after hot-water extraction of meat did not greatly influence ketonuria when it was added to a high-fat diet.

The present investigation was undertaken to elucidate the nature of the ketonuria-stimulating substance observed by Marks & Young (1939) to be present in an extract of meat. A preliminary report of this investigation has already been published (Stewart & Young, 1952).

METHODS

Chemical reagents

2:4-Dinitrophenylhydrazine. This compound (Eastman Kodak Inc. or British Drug Houses Ltd.) was dissolved in 2N-HCl to give a 0·2% (w/v) solution. This solution was repeatedly extracted with purified CCl_4 until the extinction of the extract was less than 0·07 (*d* 1·4 cm. at 415 mμ).

Carbon tetrachloride. This was washed for 24 hr. with running tap water and redistilled.

Calcium zinc-β-hydroxybutyrate. The DL sodium salt of this compound (Hopkin and Williams Ltd.) was converted into the free acid by acidification of a solution and continuous ether extraction. The residue after evaporation of the ether was treated according to the procedure described by Shaffer & Marriott (1913–14). The primary precipitate was crystallized from 95% ethanol and twice recrystallized from water. The product was dried *in vacuo* over P_2O_5.

Periodate reagent. Sodium metaperiodate (6·40 g.) was dissolved in water and filtered through a glass filter (porosity 4) and made up to 100 ml. Calculated as the trihydrate, the material was 88% pure. Titration in acid indicated that the bulk of impurity behaved as iodate, a substance which is innocuous in periodate oxidation.

Methods of estimation

Ketone bodies. Urine was filtered immediately before analysis, samples of not more, and usually less, than 1 ml. being used. Urinary acetone plus acetoacetate was estimated by the method of Barkulis & Lehninger (1951) with the procedure for extraction described below. Urinary β-hydroxybutyric acid and total ketone bodies were estimated by the method of Greenberg & Lester (1944) modified in the following way. After oxidation had been effected under the conditions described by these authors, 3 ml. of the product was placed in a 25 ml. glass-stoppered cylinder. The residual dichromate was discharged with sulphite and the mixture treated successively with 5 ml. of 2:4-dinitrophenylhydrazine reagent and 10 ml. of purified CCl_4. Extraction, the conditions for which are important, was carried out on a Townson and Mercer Ltd. laboratory shaker at 280–300 strokes/min. for 20 min. After the phases had separated, the supernatant was removed by suction and the solvent washed twice with 15 ml. of water by vigorous shaking for 15 sec. The water was replaced with 10 ml. of 0·5N-NaOH and the solvent re-extracted for 15 min. on the shaker under the conditions described. The supernatant was removed and the extinction of the solvent estimated in a 1 cm. cell at 415 mμ by the Beckman model DU quartz spectrophotometer with a slit width of 0·1 mm. A standard estimation on calcium zinc β-hydroxybutyrate was carried through with each set of analyses.

The Rothera qualitative test for urinary ketone bodies was carried out according to the method described by Harrison (1947). Blood samples were prepared and analysed by the method of Greenberg & Lester (1944).

Urinary keto acids. These were determined by the direct procedure of Friedemann & Haugen (1943).

Blood sugar. This was estimated by the colorimetric method of Somogyi (1945).

Liver glycogen. This was estimated by the method of Good, Kramer & Somogyi (1933) on tissue quickly removed from an animal given a large fatal dose of Nembutal intraperitoneally.

Liver fat content. Slices from representative parts of the liver (total of about 300 mg. wet wt.) were weighed and dried to constant weight in tared containers for the determination of the ratio of wet wt./dry wt. The remainder of the liver was ground with twice its weight of anhydrous Na_2SO_4 and extracted in a Soxhlet apparatus for 4 hr. with methanol and for 8 hr. with ether. The combined extracts were evaporated to dryness *in vacuo*, the residue was taken up in light petroleum and filtered and the filtrate evaporated to dryness in a tared container which was weighed.

Spectrophotometry. Except where otherwise indicated, colorimetric estimations were carried out with a Unicam model SP. 350 spectrophotometer. Infrared-absorption spectra were obtained by means of a Perkin–Elmer double-beam instrument, model 21.

Animals

Hooded male rats weighing 150–160 g., of a strain bred in the Laboratory over a period of many years, were used in these experiments. In a few preliminary experiments similar results were obtained whether intact rats or alloxan-diabetic rats were used and all the observations subsequently made and recorded in the present communication were obtained in experiments with intact rats.

High-fat diet

In agreement with Burn *et al.* (1944) we found that the gradual addition of fat to the diet of the rat did not induce ketosis, whereas if the rat which had been eating normal food was suddenly transferred to a high-fat diet (e.g. one containing 70% of margarine) a substantial ketonuria developed. Nevertheless, if the animal continued to eat a suitable high-fat diet, the ketonuria steadily fell and in 15–30 days the urine became free from ketone bodies as judged by the Rothera test. In preliminary experiments it was found that a diet consisting of 70% of margarine and 30% of raw horse muscle produced a severe ketonuria, whereas one consisting of 70% of margarine, 6% of casein, salts and water did not. Accordingly a diet containing 70% of margarine and 6% of casein, together with 5% of salt mixture (Dunn Nutritional Laboratory salt mixture from British Drug Houses Ltd., London, based on that described by McCollum & Davis, 1915) and 19% of water,

was adopted as the standard high-fat diet in the present investigation. The calorie value of 100 g. of this diet, together with the caloric contribution of each principal component, is given in Table 1 together with data for the normal diet (diet 41, Bruce & Parkes, 1949) used for control animals.

The fat of the diet was Ministry of Food 'Special Margarine' consisting of about 83% of fat, 0·1% of protein and 15·5% of water, and containing 9·8% of linoleic acid and 0·2% of linolenic acid in addition to saturated even-number fatty acids from C_6 to C_{24} and some mono-unsaturated fatty acids. 'Low vitamin casein', supplied by Genatosan Ltd., Loughborough, Leics, was used in most instances. In a few experiments the casein was replaced by heat-coagulated dried horse muscle prepared by dropping minced fresh horse muscle into boiling water and maintaining it at 80–85° for 3–4 min., followed by straining through a sieve and air- and oven (100–105°)-drying. The dried residue was ground in a mechanical mortar into a soft fibrous powder.

The diet was prepared by adding the solid components to the margarine in a vessel which was warmed until the margarine was soft but not liquid. The water, salt mixture and casein were then blended into the margarine by vigorous beating and the mixture was allowed to set into a solid paste, which was stored at 2° in tightly covered containers. Diet sufficient for a week to 10 days was prepared at one time and no tendency to develop rancidity was observed. For feeding to the rats the diet was packed into glazed earthenware dishes with overhanging edges. These in turn were placed in porcelain pots, a procedure which was found to minimize the scattering of food.

In addition to this food, with water *ad libitum*, a supplement of vitamins was given (Table 2). Vitamins A and D were present in more than adequate quantities in the margarine used. Parenteral administration of vitamins E and K had no effect on the weight curves or condition of the fat-fed animals and these vitamins were not always included in the diet. Inositol, in small and large doses, had no detectable effect on the weight curves or condition of the test animals and was omitted from the vitamin mixture because it was found to be antiketogenic (see also Wiebelhaus, Betheil & Lardy, 1947; Beatty & West, 1951). The other vitamins were prepared in two stock solutions, *A* and *B* (Table 2), which were mixed immediately before presentation to the rat. After a few days the supplement was readily taken by mouth. The composition of the mixture was so adjusted that approximately ten times the minimum requirements of the vitamins (Williams, Eakin, Beerstecher & Shive, 1950) were received daily. This large dose was considered desirable in order to prevent deficiencies arising from reduced intestinal absorption, or diminished intestinal synthesis, which might result from the high-fat content of the intestine. Nicotinamide, not normally required by the rat, was included because of the low-protein content of the diet.

The common B-group vitamins were obtained from Roche Products Ltd., Welwyn Garden City, Herts. Folic acid was obtained from Lederle Laboratories, London, and vitamin B_{12} was prepared by Glaxo Laboratories, Greenford, Middlesex.

Table 1. *Calorie contribution of the principal constituents of the high-fat and normal diets*

In the calculation of the composition of the high-fat diet no allowance has been made for the small amount of skim milk that may have been added to the margarine during manufacture. The carbohydrate calories of the high-fat diet are calculated from the glycerol content of the margarine.

	kcal./100 g. of diet		kcal. contribution (%)	
Constituent	High-fat diet	Normal diet	High-fat diet	Normal diet
Carbohydrate	22	198	4	67
Fat	499	41	93	14
Protein	16	58	3	19
Total	537	297	100	100

Table 2. *Water-soluble vitamin mixture for high-fat diet*

The total daily dose was dissolved to give 0·7 ml. of each of solutions *A* and *B*, which were mixed immediately before feeding.

Vitamin	Daily dose (mg.)
Solution *A*	
Thiamine	0·112
Riboflavin	0·140
Nicotinamide	0·700
Pantothenic acid	0·252
Pyridoxine	0·112
Biotin	0·014
Folic acid	0·238
Vitamin B_{12}	0·0007
Solution *B*	
p-Aminobenzoic acid	10·0
Choline	10·0

Normal diet

This was diet 41 of Bruce & Parkes (1949), usually made into a paste with water.

EXPERIMENTAL AND RESULTS

Effect of prolonged feeding of the high-fat diet on the normal rat

After a short time the rats ate the high-fat diet readily, for up to 2 years in some instances. From Table 3 the voluntary calorie intakes of rats receiving the high-fat diet and of those eating normal diet are seen to be very similar, but the mean daily gain in weight of the rats receiving the high-fat diet was subnormal (Table 3).

Most of the animals on the high-fat diet had greasy coats, and a loss of hair on the ventral parts of the trunk was sometimes observed. A few of the animals exhibited a peculiar deficiency syndrome, with lesions on the snout, trunk and tail, which was unresponsive to vitamin therapy but which usually cleared up if a small amount of carbohydrate was given to the animal. The rats which developed these lesions were not used in the studies on ketogenesis.

Table 3. *Calorie intakes of rats fed on the high-fat and normal diets*

Results given are mean values together with, in some instances, the standard errors of the means.

Diet	No. of rats	Period of observation (days)	Mean calorie intake (kcal./kg. of rat/day)	Mean gain in weight (g./day)
High-fat	5	21	439±36·2	0·51
Normal	6	9	420±16·5	5·0

Table 4. *Approximate water balance of rats on the high-fat and normal diets*

Diet	No. of rats	Period of observation (days)	Water drunk (ml./day)	Water present in food (ml./day)	Water calc. to be formed catabolically (ml./day)	Total intake of water (ml./day)	Urine excreted (ml./day)	Calc. inevitable water loss (ml./day)
High-fat	6	24	20·5	1·9	6·0	28·4	12·4	16·0
Normal	4	25	13·0	13·1	7·9	34·0	9·6	24·4

Table 5. *Analysis of blood and urine of non-fasting rats receiving the high-fat and normal diets*

Results given are the mean values together with the standard errors of the means. Figures in parentheses are the numbers of observations made.

Constituent	Unit of measurement	Value for rats on: High-fat diet	Value for rats on: Normal diet	Probability that difference is not significant
(*a*) Blood				
1. Sugar	mg./100 ml.	85±2·0 (7)	126±17 (6)	<0·01
2. Ketone bodies	Equivalent to mg. of β-hydroxybutyric acid/100 ml.	10·50±0·83 (16)	1·05±0·26 (4)	<0·001
(*b*) Urine				
1. Keto acids	Equivalent to mg. of pyruvic acid/24 hr.	0·78±0·21 (35)	1·80±0·24 (22)	<0·001

Marks & Young (1939) remarked on the small volume of urine excreted by dogs consuming a high-fat diet, and Funk & Zefirow (1932) and Bornstein & Nelson (1949) made similar comments about the effects of a high-fat diet on the urine volume of the rat. We found (Table 4) that the water balance of the rats fed on our high-fat diet was not substantially abnormal. Since the observations on the experimental and control groups were not made simultaneously and since the ambient humidity was not controlled, the apparent greater urine excretion and smaller inevitable water loss of the rats receiving the high-fat diet is probably not of significance. In any case the urine excretion of rats on our high-fat diet was certainly not abnormally low.

The blood-sugar level of the rats on the high-fat diet was significantly subnormal (Table 5), whereas the blood ketone-body level appeared to be raised (Table 5). The urinary excretion of α-oxo acids was low in the rats fed on the high-fat diet (Table 5). The rats on the high-fat diet after a period of ketonuria usually excreted urine free from acetone and acetoacetic acid, as indicated by a negative Rothera test, the total daily ketone-body excretion (expressed as mg. of equivalent β-hydroxybutyric acid) being 0·47 ± 0·22. A few of the animals on the high-fat diet excreted urine which gave a positive Rothera test indefinitely, and these were excluded from those used in the studies on ketogenesis. It may be noted that when the casein of the high-fat diet was replaced by heat-coagulated dried horse meat, the mean ketone-body excretion rose more than 20-fold, with a possible slight diurnal variation in excretion, about 40%, of the total for 24 hr. being excreted between 10 a.m. and 10 p.m. When glucose, pyruvate and glycerol, and probably other anti-ketogenic substances, were fed to the rats receiving the high-fat diet in which casein had been replaced by dried horse meat, a sharp anti-ketogenic action was observed. This effect is illustrated for glycerol in Table 6.

Simultaneous estimation of blood and urinary ketone bodies of the rats receiving the standard high-fat diet, and of those given a similar diet in which the casein had been replaced by dried horse meat, yielded an opportunity of assessing the approximate renal threshold for ketone bodies in the rat receiving a high-fat diet. The results (Table 7) suggest a renal threshold of 20–30 mg./100 ml., a value similar to that observed for normal rats treated with pituitary extract by Shipley & Long (1938). Although other factors may alter the renal threshold for ketone bodies (Mirsky, 1938;

MacKay & Wick, 1939) the feeding of a high-fat diet is apparently not one of them.

The fat content of the livers of our rats receiving the high-fat diet was not very, though significantly, high (Table 8); the glycogen content was subnormal (Table 8). The size of the liver and its content of fat-free solids were within normal limits (Table 8).

Procedure for testing ketonuria-stimulating activity of meat extracts

The rats used for the detection of ketonuria-stimulating activity received the high-fat diet for at least 30 days before the test began and in many instances for much longer. The animals were kept in metabolism cages similar to those described by Ackroyd & Hopkins (1916). Urine was collected at the same time each day and analysed at once. No urinary preservatives were used.

Animals fed on the high-fat diet, whose urinary Rothera test had been negative for at least 10 days, were dosed by mouth with the material under test. If the substance to be tested was a solid it was blended into the vitamin mixture; if it was a liquid it was given by stomach tube or dropwise into the back of the pharynx. It was customary to dose animals at the same time each day and in a single assay either two or three doses were given on successive days. Ketonuria usually appeared within 24 hr. of the completion of dosing with active material, but on some occasions did not appear for several days. Such a delay in response appeared to be a characteristic of particular animals and not of the test preparation, and may result from a delay in absorption from the gut.

Although this procedure is qualitative, experience has shown that the intensity and duration of the response may be used to give roughly quantitative information.

Preparation of ketonuria-stimulating extracts of meat

Fresh horse muscle was freed from excess of fat and fascia and minced. The mince was freeze-dried in batches of about 10 kg. and stored at 2°. The freeze-dried cake was broken into a coarse powder and extracted in 250 g.

Table 6. *Effect of feeding glycerol on ketosis of rats receiving a high-fat diet containing dried horse meat as the source of protein*

Day of experiment	Dose	Blood ketone body [mg. (as β-hydroxybutyrate)/100 ml.] at 10.00 a.m.	Urinary excretion [mg. (as β-hydroxybutyrate)/24 hr.]
1	—	45·1	34·6
3	—	40·8	49·8
6	400 mg. of glycerol *per os* at 10.15 a.m.	37·0	33·2
7	—	8·2	1·2
8	—	33·0	4·9
9	—	—	26·1
10	—	51·8	44·3

Table 7. *Blood and urine ketone-body contents for rats fed on a high-fat diet containing either casein or dried horse meat as the source of protein*

Dietary protein	Range of blood ketone-body contents [mg. (as β-hydroxybutyric acid)/100 ml.]	Number of observations	Urine ketone-body content [mg. (as β-hydroxybutyric acid)/24 hr.]
Casein	0–4·9	1	0·21
Casein	5·0–9·9	4	0·39
Casein	10·0–14·9	11	0·73±0·66
Casein	15·0–19·9	7	1·00±1·0
Casein	20·0–24·9	5	1·06±0·47
Horse meat	30·0–34·9	1	12·5
Horse meat	35·0	6	63·5

Table 8. *Composition of liver tissue from rats receiving high-fat and normal diets*

Results given are the mean values together with the standard errors of the means. The figures in parentheses are the numbers of observations made.

Diet	Size of liver (g./100 g. of rat)	Amount in liver tissue (g./100 g. of liver tissue)			
		Water	Glycogen	Fat	Fat-free solids
High-fat	3·65±0·55 (13)	69·1±2·9 (8)	1·41±0·54 (8)	7·26±1·51 (13)	23·8±3·9 (8)
Normal	3·47±0·23 (8)	72·7±0·5 (8)	4–5	4·80±0·25 (8)	22·5±0·75 (8)
Probability (P) that difference is not significant	>0·1	>0·1	—	<0·001	>0·1

portions with approx. 10 times its weight of butan-1-ol containing 10% (v/v) of water. Extraction was carried out with slow shaking for 12 hr., the solvent decanted and the extraction repeated four times. The extract contained the activity, and the residue, freed of solvent (dried extracted horse muscle), could be used as a source of protein in a non-ketogenic high-fat diet. The pooled extract was filtered, concentrated to about 250 ml. by vacuum-distillation at about 30°, washed twice with hot water to remove salts and filtered again. The filtrate, in a precipitating jar, was cooled to −10° overnight. This treatment converted the solvent into a semi-solid mass, which was thawed at 2°. The bulk of the brown supernatant was removed by suction and the tan-coloured residue was separated by centrifuging at 2°. This residue was taken up in warm butan-1-ol and cooled to 5°; an almost pure white solid deposited and the supernatant was discarded. This preparation was taken through the process of fractional crystallization shown in Fig. 1. Testing of fractions I, II (250 mg./day thrice) and IV (200 mg./day thrice) showed that fraction I possessed a trace of activity, II was inactive and IV produced a ketonuria which persisted for 14 days. Fraction III appeared to possess little or no activity. Tests on the concentrate from the butanol mother liquors were unsatisfactory because of difficulty in handling the viscous preparations and because of the anorexia they produced in test animals.

Some variation in ketonuria-stimulating activity was encountered among the 16 preparations of this sort obtained from different batches of horse meat. One preparation which had the expected melting point, and which was obtained in the expected yield, was inactive for no apparent reason. A typical response is shown in Fig. 2. In general 300–600 mg. of lipid was needed to induce a brisk response, and this amount of lipid corresponds to 500–1000 g. of fresh horse meat. Since the consumption by the rat of a diet consisting of 70% of margarine and 30% of raw horse meat and consumed at the rate of 10–15 g./day induces a similar ketonuria, it appears that the ketonuria-inducing activity of the lipid represents only a small proportion of the total activity of the raw meat.

Purification of the active substance. Some preparations were subjected to further purification by countercurrent distribution. This was carried out in graduated 150 ml. separating funnels lubricated with graphite. The composition of the solvent system selected (*n*-hexane–propan-1-ol–formamide, 40:40:10) was chosen as the result of preliminary investigations in which this system was found to yield the most suitable partition coefficient with undistributed material. The solvents, which had been freshly

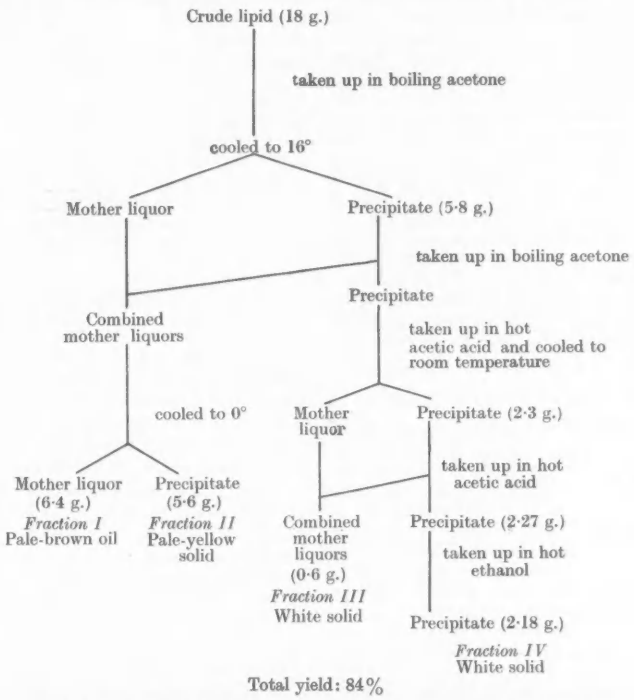

Fig. 1. Procedure for fractional crystallization of crude muscle lipid.

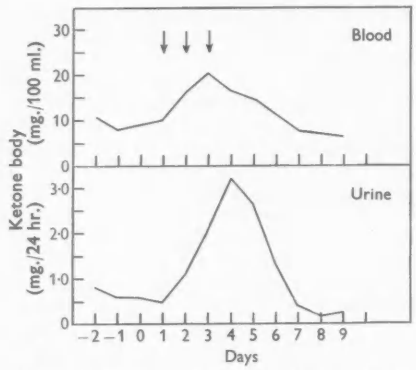

Fig. 2. Effect of muscle lipid on the blood ketone-body level and the urinary excretion of ketone bodies. Each vertical arrow corresponds to a dose of 100 mg. of lipid.

redistilled, were equilibrated at room temperature, which was maintained during the distribution at 20°±1. Solute (200 mg. when the volume of each phase was 25 ml.) was dissolved in the upper phase of funnel no. 1. The transfers (23) were carried out over a period of about 8 hr. On completion of the distribution the two-phase system in each of the separating funnels was treated with 50 ml. of glass-distilled water. The aqueous phase containing the formamide and some of the propanol was separated and the hexane fraction was washed with an additional 50 ml. of water. No solute could be demonstrated in these washings. The residual volume of solvent in each funnel was recorded. The weight curve for the distribution of the lipid was obtained by evaporating samples (2 ml. × 2) in tared glass shells (Craig, Hausmann, Ahrens & Harfenist, 1951) on a water bath, the solvent-free dishes being dried to constant weight. Colorimetric estimation of the ester bonds in the material was based on the formation of a coloured ferric hydroxamate, the procedure described by Hill (1946, 1947) being used. The colour was estimated at 520 mμ. Undistributed material was used in the construction of the calibration curve. Theoretical curves were calculated as described by Craig & Craig (1950). The total recovery from the distribution was 71%.

Chemical examination of ketonuria-stimulating extracts of meat

Saponification of 216·8 mg. of lipid was effected by heating with 20 ml. of ethanolic 90·12 mN-NaOH (CO_2-free) on a boiling-water bath for 105 min. under conditions which excluded CO_2. Titration of the residual alkali was carried out with standard 0·1N-H_2SO_4 in an atmosphere of N_2. A control flask without lipid was put through the same procedure. The saponification mixture was evaporated to dryness on a boiling-water bath under a jet of N_2, and drying was completed in a vacuum desiccator over $CaCl_2$. The residue was taken up in warm water and transferred with washings to a small Kutscher–Steudel extractor and acidified with dilute H_2SO_4 (final volume 50 ml.). The control material was treated in the same way. The precipitated fatty acid was removed by extraction with light petroleum (b.p. 40–60°) and the residue transferred quantitatively with washings to a 100 ml. volumetric flask (final volume about 90 ml.). Periodate reagent (4 ml.) was added and the experimental and control flasks were made up to volume. Oxidation was allowed to proceed in the dark for 2 hr. at room temperature. Portions of the mixture (10 ml.) were then treated with 5 ml. of 0·5 M-phosphate buffer, pH 7·4, and 5 ml. of 40% (w/v) KI and titrated with 0·1N-arsenite (see Bell & Greville, 1950) in order to estimate unused periodate. The end-point was sharpened by the use of polyvinyl alcohol as an indicator (Miller & Bracken, 1951). Titrations on blank and experimental solutions were carried out in duplicate.

The formic acid formed by periodate oxidation was estimated by titration after steam-distillation in the apparatus of Markham (1942). The periodate oxidation mixture (15 ml.) was treated with 0·5 ml. of redistilled, acid-free, ethylene glycol to destroy the residual periodate and the mixture was made up to 25 ml. with 10% (w/v) solution of $KHSO_4$. Portions of this solution (5 ml.) were steam-distilled until 65 ml. of distillate had been collected. Titration was carried out under N_2 with 9·37 mN-NaOH dispensed from a Conway microburette, phenol red being used as indicator. The titration value for a blank experiment was subtracted from the result. A standard solution of formic acid gave a value of 98·3–98·6% of that expected, by this method.

The formaldehyde formed by periodate oxidation was estimated by a method adapted from that of Bell & Greville (1950). The periodate oxidation mixture (10 ml.) was treated in a 50 ml. beaker with 3 ml. of N-HCl and 5 ml. of 1·2 M-potassium arsenite. The solution was then acidified with sufficient HCl to produce a yellow colour with bromocresol green (3 drops of 0·1%, w/v, in ethanol). The colour was then adjusted with M-sodium acetate to a blue-green. Dimedone (250 mg. in 2 ml. of ethanol) was added to the mixture, which was digested for 2 hr. on a boiling-water bath and then allowed to stand for 3½ hr. at room temperature. The deposited derivative was collected in a tared sintered-glass thimble, washed with 75 ml. of water, dried *in vacuo* overnight over P_2O_5 and weighed.

Chemical properties of the ketonuria-stimulating substance

Composition. The most active preparation (fraction IV) was pure white, crystalline, insoluble in water but soluble in lower alcohols (more so in butanol than in methanol), acetone, warm (40°) acetic acid, light petroleum, benzene and ether. The melting point was relatively sharp and ranged from 52° to 54° (uncorrected) in different preparations. Sodium fusion revealed no trace of nitrogen, sulphur or halogen and a spark spectrum (Johnson Matthey and Co., London) failed to reveal significant quantities of metallic compounds. Microanalysis (Weiler and Strauss, Oxford) gave C, 74·9–76·5; H, 11·7–12·8. Calc. for tristearin C, 76·2; H, 13·0; for tripalmitin C, 75·4; H, 12·9%.

With the cooling-curve method of Reilly & Rae (1948) a molecular weight of 650 ± 50 was obtained. Saponification of 100 or 200 mg. of the lipid gave

a saponification number of 208·3, which corresponds to a molecular weight of 808·3 if a completely esterified trihydric alcohol is assumed. If the alcohol is glycerol then calculation shows that 11·42 mg. is liberated from 100 mg. of lipid.

The saponification mixture was freed from fatty acids by acidification with dilute H_2SO_4 and extraction with light petroleum, and treated under controlled conditions with sodium periodate. The periodate consumption and the formaldehyde and formic acid production were estimated in order to estimate glycerol set free. When the formic acid value was corrected for the small amount which was not volatile in steam under standard conditions the formaldehyde/formic acid ratio was 2·008, almost precisely that expected for glycerol. In Table 9 the glycerol content of the lipid, as determined by a number of methods, is shown. The small difference between that deduced from the saponification number and those based on the other methods of analysis is probably due to the presence of a trace of free acid in the lipid. The presence of a trace of acid could be demonstrated by extraction with dilute $NaHCO_3$ or by steam-distillation.

Table 9. *Glycerol content of muscle lipid determined by different methods*

Method	Glycerol from 100 mg. of lipid (mg.)
Saponification	11·42
Periodate consumption	11·23
Formic acid (corrected)	11·24
Formaldehyde	11·26

The presence in the saponification mixture of no polyhydric alcohol other than glycerol was demonstrated by the paper-chromatographic method of Hough (1950).

Since the iodine number of several preparations was 6–8 the fatty acids present appeared to be essentially saturated.

Spectroscopic examination. No absorption of light of wavelength above 240 mμ was observed for a light-petroleum solution of lipid (1 mg./ml.). The infrared-absorption spectra of two typical preparations are shown in Fig. 3. Comparison of the spectra with the data of Shreve, Heether, Knight & Swern (1950, 1951*a*, *b*) shows that the general shape of the spectrum corresponds with that reported for saturated triglycerides, but the presence of additional bands, at 10·86, 11·25, 11·93–11·99 and 13·77 μ, suggests the existence of an oxirane (ethylene oxide) ring, a suggestion which is supported by the presence of a complex of small peaks at 7·09, 7·27, 7·69, 7·95, 8·21 and 8·37 μ. A band at 10·31 μ is generally considered to be related to a *trans* double bond.

Homogeneity. The homogeneity of a triglyceride is difficult to establish unequivocally. Counter-current distribution was applied to the problem

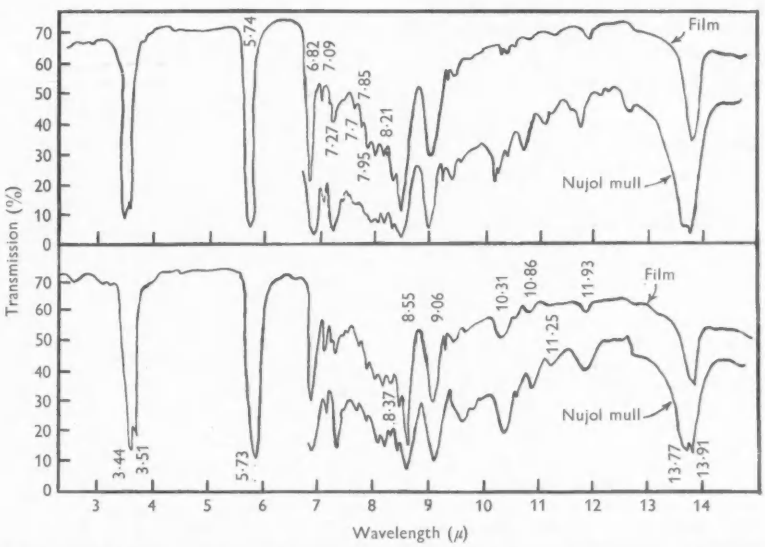

Fig. 3. Infrared-absorption spectra of two samples of horse-muscle lipid. The upper curve in each case was derived from a solid film of lipid and the lower comes from a Nujol (liquid paraffin) mull.

but a difficulty in the interpretation of the results arose from a change in the volume of the phases with changing concentration of solute (see also Olley, 1953). In Fig. 4 the distribution of the horse-muscle lipid in *n*-hexane–propan-1-ol–formamide is shown. Gravimetric and colorimetric estimation of the solute in each separating funnel were both made with the object of demonstrating that the mass peak corresponds to a single type of chemical species. If preparations at the peak of the curve consisted of a mixture of hydrocarbon, glycerol ether and glyceride, the weight curve would not correspond to the curve based on ester-group analysis. The curves indicate that the principal component is a single type of chemical species.

Since the volumes of upper and lower phase did not remain constant throughout the distribution, the conventional method of binomial analysis could not be applied. If it is assumed that the partition coefficient does not vary during the course of the distribution and a record is kept of volume changes in upper and lower phases (this can easily be done by using graduated separating funnels), it is possible to apply a correction to each term of the binomial after each transfer for the altered volume relationships, and in this way a 'corrected' theoretical curve can be obtained. The greatest volume change was found to occur between the sixth and tenth transfers, and thereafter the conditions in the first twelve funnels remained approximately constant. To calculate the theoretical curve therefore a normal distribution with an empirical selected partition coefficient was calculated for the first six transfers. Thereafter the ratio of upper to lower phase prevailing at the end of the distribution (after twenty-four transfers) was introduced to permit the calculation of the absolute amount transferred with each movement of solvent. The procedure involves the calculation of 189 terms with each selected partition coefficient. In Fig. 5 the results of the calculation are shown and it can be seen that when a partition coefficient of 0·45 is used a curve closely corresponding to the observed curve is obtained. The skewing of the experimental curve does not therefore necessarily indicate that the material distributed was a mixture.

DISCUSSION

The evidence presented suggests that the ketogenic material isolated from horse muscle is a triglyceride. Since it is active in small doses in an animal receiving a large amount of triglyceride in its diet, the extract cannot be ketogenic solely or mainly by virtue of its ability to give rise to ketone bodies. Some fraction of the glyceride, or a substance contaminating, must be an active ketogenic material. A similar material can be obtained from calf intestinal mucosa (Stewart & Young, 1952). Although a crucial test of homogeneity has not been found, such evidence as is available suggests

Fig. 4. Countercurrent distribution of muscle lipid in *n*-hexane–propan-1-ol–formamide (40:40:10) system; 200 mg. of solute was distributed in the system. The volumes of the upper and lower phases at the beginning of the distribution were 25 ml. ▲---▲, Distribution by weight; ●—●, distribution of ester bonds on corresponding ordinate scale.

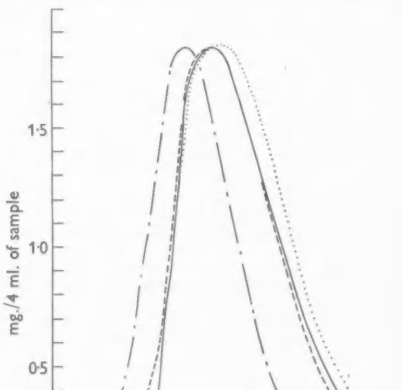

Fig. 5. Calculated theoretical curves, corrected for changes in phase volume, on the basis of three different assumed values of the partition coefficient (*K*). —, Experimentally determined curve; —·—, *K* 0·35; – – –, *K* 0·45; · · ·, *K* 0·50.

that our best preparations are unlikely to be contaminated with material other than glycerides. Glycerol is highly anti-ketogenic (cf. Table 9), and the ketopoietic properties of the glyceride are almost certainly to be attributed to the fatty acid part of the substance.

Consideration of the results of microanalysis and of the infrared-absorption spectrum suggests that one or more oxygen atoms may be attached to the fatty acid chains. The reaction of 2:4-dinitrophenylhydrazine with the lipid reported earlier (Stewart & Young, 1952) may have been with an oxygen atom of this sort, or possibly with an ester group to form a hydrazide. The dinitrophenylhydrazine derivative was difficult to purify and its preparation, like that of the benzylidene derivative, has not been consistently reproducible.

The physiological activity of partially oxidized fatty acids has not been studied in detail. In most of the reported experiments on the feeding of rancid fats the effects observed were referable to destruction of the vitamins in the diet. In our experiments there was no evidence of known vitamin deficiency. Kaunitz (1953) has studied the effect of the administration of rancid fats to rats under conditions which he believes precludes the complications which result from vitamin deficiency. He showed that these materials were absorbed from the gut and that they interfere with growth but no evidence was provided to elucidate the nature of the metabolic disturbance produced by the rancid fat in his experiments.

SUMMARY

1. Evidence has been provided for the existence of a ketonuria-stimulating substance which can be extracted from horse muscle.

2. An animal suitable for the detection of such a ketopoietic material is the normal rat given a diet rich in fat. Some of the physiological characteristics of this test animal have been described.

3. A method for the isolation of the ketopoietic substance from horse muscle has been evolved and evidence has been provided which suggests that the isolated material is homogeneous.

4. Chemical examination indicates that the isolated material is a triglyceride, and some evidence has been provided that a fatty acid chain in the glyceride is in a partially oxidized state.

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The Reactivation of Phosphorylated Chymotrypsin

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Chymotrypsin, like most other enzymes with esterase activity, is inactivated by combination with organophosphates (Balls & Jansen, 1952). The inactive phosphorylated enzyme is relatively stable in water but hydroxylamine and picolinohydroxamic acid will restore the activity by nucleophilic displacement of the enzyme from the phosphoryl residue (Cunningham, 1954; Jandorf, Crowell & Levin, 1955). The only reported kinetic study of this process is by Cunningham (1954) on reactivation with hydroxylamine, but his conclusions as to mechanism are open to criticism as, at best, only about 30% of the original enzymic activity could be recovered. The present paper describes the complete reactivation of phosphorylated chymotrypsin by oximes and hydroxamic acids. The results necessitate some modification of views on the mechanisms for chymotryptic hydrolysis which have been proposed recently (Cunningham, 1957; Dixon & Neurath, 1957*a*; Davies & Green, 1958).

EXPERIMENTAL

Materials

Commercial chymotrypsin (The Armour Laboratories) was dialysed against running tap water for 24 hr. to remove $MgSO_4$, and was then freeze-dried. A stock solution of chymotrypsin (50 mg.) in 0·1 M-KH_2PO_4 (10 ml.) was stored in a refrigerator.

N-Acetyltyrosine ethyl ester was prepared from commercial tyrosine ethyl ester by Fischer's method (1904). Hydroxyiminoacetone, salicylohydroxamic acid and picolinohydroxamic acid were prepared as described by Freon (1939), Jeanrenaud (1889) and Hackley, Plapinger, Stolberg & Wagner-Jauregg (1955). 2-*Hydroxyiminomethyl-N-methylpyridinium methanesulphonate*, m.p. 155° (Found: N, 11·8. $C_9H_{12}O_4N_2S$ requires N, 12·1%), was prepared by boiling 2-hydroxyiminomethylpyridine with methyl methanesulphonate in benzene.

Methods

Chymotrypsin was assayed by the continuous-titration method (Schwert, Neurath, Kaufmann & Snoke, 1948), whereby the acid produced by enzymic hydrolysis of an ester substrate is titrated, as produced, with 0·1 N-NaOH. The assay mixture consisted of 0·01 M-*N*-acetyltyrosine ethyl ester (7 ml.), 0·02 M-phosphate buffer, pH 8 (1 ml.), M-NaCl (1 ml.) and enzyme (1 ml. of the stock solution after 500-fold dilution).

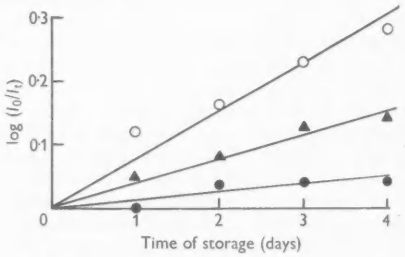

Fig. 1. Spontaneous reactivation at 25° of chymotrypsin inhibited with Sarin. The inhibited enzyme was stored in acetate buffer at pH 5·1 (○), in phthalate buffer at pH 6·3 (▲), or in borate buffer at pH 8·7 (●). Fuller details of the buffer compositions are given in Table 2.

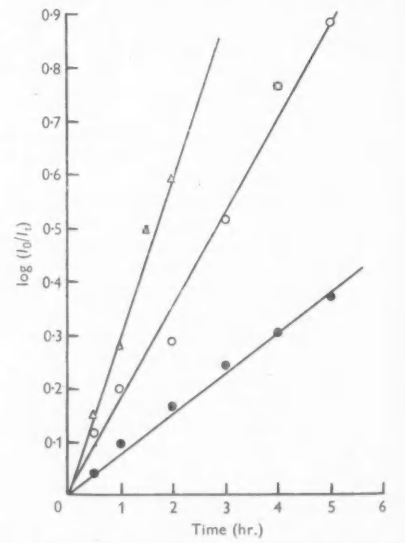

Fig. 2. Reactivation of chymotrypsin inhibited with Sarin by hydroxyiminoacetone (0·5 M) at 25° and at pH 7·5 (△), pH 8·0 (○) or pH 9·0 (●). (For details see Methods.)

The enzyme was inhibited by addition of 0·05 M-*iso*propyl methylphosphonofluoridate (Sarin) in propan-2-ol (0·2 ml.) to the stock solution of the enzyme (2·0 ml.) containing N-NaOH (0·15 ml.) to raise the pH to 7·4. Inhibition was complete within 30 min. at room temperature although there was still some unchanged Sarin present as indicated by Marsh & Neale's (1956) colorimetric method of estimation. After 3 hr. all the excess of Sarin had hydrolysed.

In the spontaneous-reactivation experiments 2 ml. of the inhibited enzyme solution (after contact with Sarin for 3 hr.) was diluted to 10 ml. with an appropriate buffer. At suitable time intervals, samples (1 ml.) were withdrawn and diluted with water to 100 ml.; 1 ml. of the diluted solution was then taken for assay. In the normal-reactivation experiments the buffer was replaced by the oxime or hydroxamic acid adjusted to the required pH. The time of contact between enzyme and inhibitor was reduced to 30 min. as any excess of Sarin would have been hydrolysed almost instantaneously on addition of the reactivator (Green & Saville, 1956). The 100-fold dilution before assay prevented any interference by the reactivator or buffer with the assay. The apparent rate constants were calculated graphically by use of the formula

$$k = \frac{2{\cdot}3}{At} \log (I_0/I_t),$$

where A is the concentration of the reactivator and I_t/I_0 the fraction of the inhibited enzyme remaining after time t. Typical experiments for spontaneous reactivation and reactivation by hydroxyiminoacetone are shown in Figs. 1 and 2.

RESULTS

Preliminary experiments with some of the oximes and hydroxamic acids which were active in dephosphorylating inhibited cholinesterase (Childs, Davies Green & Rutland, 1955) showed (i) that reactivation was far slower than with cholinesterase, (ii) that *iso*propyl methylphosphonylated chymotrypsin was more readily reactivated than diethylphosphorylated chymotrypsin or di*iso*propylphosphorylated chymotrypsin, and (iii) that among oximes and hydroxamic acids which varied little in their intrinsic reactivity with Sarin itself (Green & Saville, 1956), structural variation produced only minor changes in reactivating power (see Table 1); this is in marked contrast with what was found with cholinesterase (Childs *et al.* 1955).

Accordingly, for subsequent kinetic work Sarin was chosen as the inhibitor, and hydroxyiminoacetone, which, being highly water-soluble, could be used at high concentration, was chosen as the reactivator.

Although neither diethylphosphorylchymotrypsin nor di*iso*propylphosphorylchymotrypsin appears to hydrolyse on storage in water to regenerate any free enzyme (Balls & Jansen, 1952; Cunningham, 1954), *iso*propyl methylphosphonylchymotrypsin does slowly recover some chymotryptic activity even in the absence of any specific reactivator (see Fig. 1). This recovery by spontaneous hydrolysis was examined further (see Table 2). The results are difficult to interpret kinetically as the rate is markedly affected by the buffer, but the trend is clear, namely that hydrolysis becomes faster as the solution is made more acidic. The experiments were not continued beyond 4 days, as after this time control enzyme solutions in the same buffers had begun to deteriorate, but, as described below, the

Table 1. *Rate constants for the reactivation at 25° of chymotrypsin inhibited with Sarin*

Compound	Concn. (M)	pH	k (l./mole/hr.)
Hydroxyiminoacetone	0·3–0·5	7·5	1·2
	0·2–1·0	8·0	0·96
2-Hydroxyiminomethyl-*N*-methylpyridinium methanesulphonate	0·3–0·5	7·5	0·48
Picolinohydroxamic acid	0·1	8·0	1·1
Salicylohydroxamic acid	0·05	7·4	2·9

Table 2. *Spontaneous recovery at 25° of chymotrypsin inhibited with Sarin*

Inhibited enzyme (2 ml.) in 0·1 M-KH_2PO_4 (adjusted to pH 7·4 with NaOH) was diluted to 10 ml. with buffer. The percentage of reactivation is based on a control non-inhibited enzyme sample in the same buffer.

Buffer	pH of buffer + enzyme	Reactivation after 96 hr. (%)
5 ml. of 0·2 M-sodium acetate + 3 ml. of 0·2 N-acetic acid	5·1	48
4·5 ml. of 0·1 M-Na_2HPO_4 + 3·5 ml. of 0·05 M-citric acid	5·9	47
5 ml. of 0·2 M-potassium hydrogen phthalate + 3 ml. of 0·25 N-NaOH	5·9	40
4 ml. of 0·1 M-potassium hydrogen phthalate + 4 ml. of 0·05 N-NaOH	6·3	26
6·4 ml. of 0·1 M-Na_2HPO_4 + 1·6 ml. of 0·1 M-NaH_2PO_4	7·4	36
2 ml. of 0·2 M-Na_2HPO_4 + 6 ml. of 0·001 N-HCl	7·8	23
5 ml. of 0·2 M-Na_2HPO_4 + 3 ml. of 0·01 N-HCl	8·0	24
5 ml. of 0·2 M-sodium diethylbarbiturate + 3 ml. of 0·12 N-HCl	8·0	<10
5 ml. of 0·2 M-borax + 3 ml. of 0·2 N-HCl	8·7	<10

remaining inhibited enzyme could be reactivated with hydroxyiminoacetone up to the control activity levels.

Table 3 shows the linear dependence on concentration of the rate of reactivation of inhibited chymotrypsin by hydroxyiminoacetone at constant pH. At the pH of these experiments (pH 8) about one-third of the oxime exists in the form of its sodium salt, so that as the oxime concentration is varied the total electrolyte concentration is also varied. In order to check that this did not affect the results, one experiment was carried out with 0·5M-hydroxyiminoacetone to which had been added 0·5M-KCl. As no significant change in rate was found in the presence of this additional electrolyte it was not deemed necessary to keep the ionic strength of the solutions constant in subsequent experiments. A linear dependence of rate on concentration was assumed in calculating the rate constants given in Table 1.

Table 3. *Rate constants for reactivation by hydroxyiminoacetone at* pH 8 *and* 25° *of chymotrypsin inhibited with Sarin*

Concn. of hydroxyiminoacetone (M)	Time (hr.) required for 50% reactivation	k (l./mole/hr.)
0·2	3·6	0·96
0·3	2·25 2·6	0·98
0·5	1·7 1·6 1·45	0·89
1·0	0·6 0·8	0·99

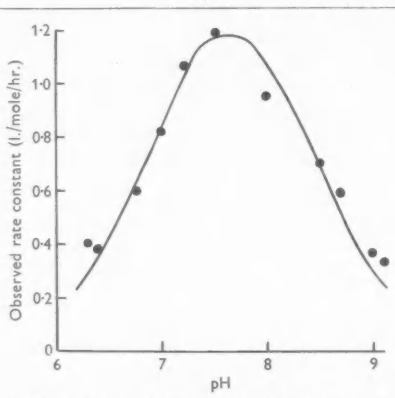

Fig. 3. Dependence on pH of the rate of reactivation at 25° by hydroxyiminoacetone of chymotrypsin inhibited with Sarin.

The effect of pH on the rate of reactivation by hydroxyiminoacetone is shown in Fig. 3. The bell-shaped curve is similar to that found for reactivation with hydroxylamine (Cunningham, 1954) and for reactivation by hydroxyiminoacetone of inhibited cholinesterase (Davies & Green, 1956), and can be interpreted in the same way. The curve superimposed on the experimental points was drawn on the assumption that reactivation is due solely to a reaction between the anion of the oxime (pK_A 8·3) and a protonated form of the inhibited enzyme with pK_A 7·0.

Cholinesterase, when inhibited with organophosphates, gradually changes from a form which can be reactivated by oximes and hydroxamic acids into one which cannot. This change is accelerated in mildly acidic solution (Davies & Green, 1956; Hobbiger, 1956). In order to see whether inhibited chymotrypsin undergoes a like change solutions of it were inhibited with Sarin at pH 7·4 and were then diluted with buffers to maintain the pH at 7·4 or to alter the pH to 5 or 8. These solutions were then stored at 25° for 4 days, after which time they were treated with hydroxyiminoacetone (0·5M) at pH 8 for 6 hr. Complete reactivation occurred in each (see Table 4).

DISCUSSION

Organophosphates react with chymotrypsin to give an inactive protein containing a single phosphoryl group per molecule (Balls & Jansen, 1952). Acidic or enzymic hydrolysis of the phosphorylated enzyme gives a number of phosphorylated peptides but the phosphorus atom is invariably found attached to the hydroxylic oxygen of a single

Table 4. *Reactivation of chymotrypsin inhibited by Sarin after storage at* 25°

Chymotrypsin inhibited by Sarin at pH 7·4 was diluted with buffers at different pH values. After storage for 4 days the inhibited enzyme solution was treated with hydroxyiminoacetone (0·5M) for 6 hr. at pH 8. Enzyme activities are given in arbitrary units. Fuller details of the buffer compositions are given in Table 2.

		Activity after 0 hr.		Activity after 96 hr.	
Buffer	pH	Enzyme control	Inhibited enzyme	Enzyme control	Reactivated enzyme
Acetate (0·1M)	5·1	200	20	186	180
Phosphate (0·1M)	7·4	203	0	163	162
Barbitone (0·1M)	8·0	177	11	177	177

serine residue (Cohen, Oosterbaan, Warringa & Jansz, 1955; Schaffer, Simet, Harshman, Engle & Drisko, 1957). More recently, inactive monoacylated chymotrypsins have been isolated (McDonald & Balls, 1957), which are similar to the phosphorylated enzymes although they are much more readily reactivated. Degradation experiments have shown that in monoacetylchymotrypsin, the acetyl group is attached to the same serine hydroxyl group as is the phosphoryl group in phosphorylated chymotrypsin (Oosterbaan & van Adrichem, 1958). This evidence, together with the similar dependence on pH of phosphorylation, of acylation and of normal substrate hydrolysis (Hartley, 1956; Gutfreund & Sturtevant, 1956; Dixon & Neurath, 1957*b*), suggests that the initial step in each case is phosphorylation or acylation of a serine hydroxyl group catalysed by the imidazole ring of a histidine residue. In normal substrate hydrolysis the acylated enzyme is assumed to hydrolyse immediately to regenerate the free enzyme. Hydrolysis of the more stable acylated enzymes can be followed kinetically (Gutfreund & Sturtevant, 1956; Dixon & Neurath, 1957*b*) and it has been shown that hydrolysis occurs more rapidly as the pH is raised and can be explained as a histidine-catalysed hydrolysis of the acylated serine.

If the mechanism of phosphorylation and dephosphorylation is identical with that of acylation and deacylation, then reactivation of the phosphorylated enzyme should occur more readily with increasing pH. In fact, as shown above, quite the reverse is true, the phosphorylated enzyme being hydrolysed less readily as the pH is increased. Furthermore, the rate of reactivation by hydroxyiminoacetone similarly falls in more alkaline solution. These results are consistent with the effect of pH on the hydrolysis of a phosphorylated imidazole group (Atkinson & Green, 1957), but not with an imidazole-catalysed hydrolysis, yet the degradation evidence seems overwhelming that the amino acid which is phosphorylated is serine, not histidine. These discrepancies can be resolved, at least qualitatively, if the acylated (or phosphorylated) active centre in its various possible states of ionization exists as shown in Fig. 4.

In neutral solution or weakly alkaline solution (about pH 8) the acylated enzyme exists primarily as an equilibrium mixture of forms *b* and *e* (Fig. 4), which are interconverted via forms *a* and *g* or *c* and *g*.

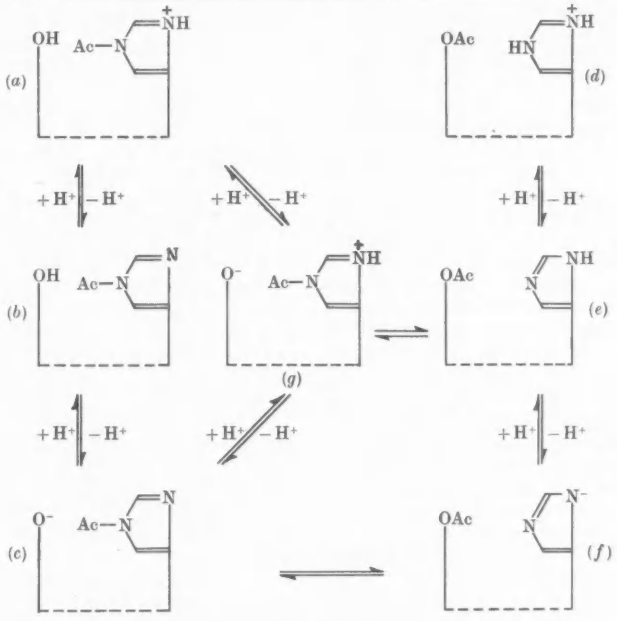

Fig. 4. The hydroxyl group is that of a serine residue, the imidazole group that of a histidine residue. These are assumed to be held in juxtaposition by the configuration of an unspecified number of amino acids. Equilibrium signs are drawn only where the two structures differ only in the migration of a single atom or group.

The predominant form is assumed to be *e*, so that on degradation the main product would be an acyl- or phosphoryl-serine. Forms *c* and *f* will occur to a significant extent only in fairly strong alkali and can be ignored under normal conditions.

In more acidic solution the equilibrium will lie mainly between *a* and *d*, *d* being assumed to be predominant. Since acylated hydroxyl groups are generally fairly stable whereas acyl imidazoles are relatively unstable it is reasonable to assume that before the acyl group can be hydrolysed away from the enzyme it must be located on the imidazole ring. Thus for hydrolysis to occur the predominant *O*-acyl form (*d* and *e*) must be converted via *g* into the *N*-acyl form (*a* and *b*). If the hydrolysis of the *N*-acyl form were more rapid than the acyl-migration reaction ($e \rightarrow g$) the effect of pH on the rate of hydrolysis would be determined by the fraction of the *O*-acylated enzyme in form *e*. This is consistent with the observed increase in the rate of deacylation with increasing pH (Gutfreund & Sturtevant, 1956; Dixon & Neurath, 1957*b*). If, in dephosphorylation on the other hand, the rate-controlling step were the hydrolysis of the *N*-phosphoryl form and not the $O \rightarrow N$ migration ($e \rightarrow g$) of the phosphoryl group, then the rate of dephosphorylation would be expected to increase and not decrease with decreasing pH, as found and as observed with di-*iso*propylphosphorylimidazole (Atkinson & Green, 1957).

This modified theory of deacylation and dephosphorylation does not necessitate any amendment to previous theories (Cunningham, 1957; Dixon & Neurath, 1957*a*; Davies & Green, 1958) that acylation and phosphorylation involve an imidazole-catalysed acylation or phosphorylation of serine. All the above reactions may be assisted or hindered by hydrogen-bonding but the evidence is insufficient for any categorical assertions as to which groups are bonded in this way.

It is becoming widely accepted that not only are individual esterases acylated and phosphorylated by what is basically the same mechanism, but also that the mechanism of phosphorylation of esterases generally is fundamentally the same. The similarity in the dependence on pH of phosphorylation of a wide range of esterases (Mounter, Alexander, Tuck & Dien, 1957) and the isolation of very similar phosphorylated peptides on the degradation of inhibited esterases (Cohen *et al.* 1955) strongly support this belief. The resemblance between inhibited chymotrypsin and inhibited cholinesterase in the dependence on pH of the rate of reactivation offers yet further support. If this idea is correct then the above picture of the phosphorylated active centre of chymotrypsin should also be applicable to cholinesterase, but phosphorylated cholinesterase, unlike phosphorylated chymotrypsin, slowly changes on storage from a form which can be reactivated to one which cannot. This change has been tentatively attributed (Jandorf, Michel, Schaffer, Egan & Summerson, 1955; Davies & Green, 1956; Hobbiger, 1956) to migration of the phosphoryl group from histidine, which is assumed to be the initial site of phosphorylation, to serine, but if the above picture is correct this explanation is untenable. An alternative possibility (W. N. Aldridge, unpublished work) is that, on storage of the inhibited enzyme, one of the alkyl groups is split off the alkylphosphoryl residue, giving a phosphate anion resistant to nucleophilic attack by the reactivator. This dealkylation may be accelerated in inhibited cholinesterase by interaction between the alkyl group and the anionic-specificity site on the enzyme which is absent in chymotrypsin. This would explain the chemically unexpected fact that conversion from the 'reactivatable' form into the 'non-reactivatable' form occurs much more rapidly with di*iso*propylphosphoryl-cholinesterase and *iso*propyl methylphosphonyl-cholinesterase than with diethylphosphoryl-cholinesterase. This theory could be proved or disproved unequivocally by comparison of the conversion rate with the rate of loss of ^{14}C from purified cholinesterase inhibited with Sarin containing ^{14}C in the *iso*propyl group.

SUMMARY

1. Oximes and hydroxamic acids will completely reactivate chymotrypsin inhibited with organophosphates, at a rate varying linearly at constant pH with the concentration of the reactivator.

2. Hydroxyiminoacetone, 2-hydroxyiminomethyl-*N*-methylpyridinium methanesulphonate, picolinohydroxamic acid and salicylohydroxamic acid have about equal potency as reactivators of chymotrypsin inhibited with *iso*propyl methylphosphonofluoridate (Sarin). This is in line with their roughly equal reactivities with Sarin itself but is in marked contrast with the great difference in their abilities to reactivate inhibited cholinesterase.

3. A bell-shaped pH–activity curve was found for reactivation by hydroxyiminoacetone of chymotrypsin inhibited by Sarin.

4. On storage at 25° for 4 days at various pH values, inhibited chymotrypsin, unlike inhibited cholinesterase, did not lose its ability to be reactivated.

5. Chymotrypsin inhibited with Sarin slowly recovers its activity even in the absence of any specific reactivator. This spontaneous recovery is accelerated by a decrease in pH.

6. A structure is proposed for the phosphorylated or acylated active centre of chymotrypsin which will explain (*a*) the isolation after degradation of

phosphorylated or acylated chymotrypsin of peptides in which the phosphoryl or acyl group is attached to a serine hydroxyl group, (*b*) the increase in the rate of hydrolysis of acylchymotrypsin as the pH is raised, and (*c*) the increase in the rate of hydrolysis of phosphorylchymotrypsin as the pH is lowered.

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Substrate Specificity of Rumen Cellulolytic Enzymes

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Cell-free preparations of rumen micro-organisms prepared by butanol extraction have been previously shown to contain at least two types of enzyme involved in the hydrolysis of carboxymethylcellulose, one of these, akin to cellobiase, being selectively inhibited by D-gluconо-1 → 4-lactone (Festenstein, 1958*a*). It has also been shown (Halliwell, 1957*a*, *b*) that the type of extract studied is without appreciable action on filter paper or cotton fibres. In the present work, specificity studies have been extended to cellophan and cellulose dextrin and the inhibitory effect of gluconolactone has been examined with reference to specific glucose production from cellulosic substrates, as well as from cellosaccharides up to cellopentaose.

EXPERIMENTAL

Substrates. Soluble cellulose dextrin was prepared from absorbent cotton wool and also from filter-paper powder (Whatman, standard grade) by solution in 72% H_2SO_4, as described by Kooiman, Roelofsen & Sweeris (1953); small amounts of insoluble cellulose dextrin were removed along with the excess of $BaCO_3$ added for neutralization of acid and the filtrates were clarified by centrifuging at 10 000 *g*. The final products, twice precipitated by 80% ethanol, gave values of 20 (filter paper) and 30 (cotton wool) for the degree of polymerization, as determined by the cuprimetric and hypoiodite methods used for determination of reducing sugar (Festenstein, 1958*a*); they were soluble in the buffered solutions used in the enzyme-assay procedure, but small amounts of dilute H_2SO_4 gave precipitates which did not dissolve on subsequent neutralization.

Cellotriose, cellotetraose and cellopentaose were gifts from the British Rayon Research Association; they were chromatographically pure and gave reducing values of 1·0, 1·18 and 1·18 respectively, expressed in terms of reduction of the Nelson–Somogyi reagent (Somogyi, 1952) by equimolecular quantities of glucose; the value obtained for cellobiose (L. Light and Co. Ltd.) was 1·12.

Sodium carboxymethylcellulose containing an average of 0·5 carboxymethyl unit per glucose residue (Cellofas B, Imperial Chemical Industries Ltd.), cellobiose (L. Light and Co. Ltd.) and cellophan (PT 300, British Cellophane Ltd.) were commercial preparations. Cellophan strips weighing 25 mg. were cut from the sheets supplied and soaked in water for at least 48 hr. before use, in order to

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remove soluble plasticizers; specimens which were subsequently dried at 105° showed an overall weight loss of 20%.

Enzyme preparations. These were produced by butanol-extraction of sheep-rumen micro-organisms centrifuged at 1500 *g*, as described previously (Festenstein, 1958*a*). It was established that three extractions recovered about 50% of the carboxymethylcellulase activity of the intact rumen micro-organisms, measured by incubation in the presence of toluene.

Enzyme assay. Substrates were incubated in 0·02 M-citric acid–0·04 M-Na_2HPO_4 buffer, pH 6·1 (occasionally pH 5·0), at 37° as described previously (Festenstein, 1958*a*, *b*). Solutions were deproteinized before estimation of reducing sugar (by the cuprimetric method) and also before the specific determination of glucose by glucose oxidase (Huggett & Nixon, 1957), where high enzyme concentrations interfered with colour development; 1 ml. each of 0·3 N-$Ba(OH)_2$ and 5% (w/v) $ZnSO_4$ were found sufficient to precipitate the protein in 0·8 ml. of enzyme. Toluene (0·1 ml./ml. of aqueous incubation mixture) was added for assay in the presence of micro-organisms and also for assay of cell-free preparations incubated for longer than 24 hr. (0·2 ml./ml.).

Chromatography. The procedures used were as described before (Festenstein, 1958*a*).

RESULTS

Effect of pH *on enzyme activity.* The optimum pH varied with substrate and also with concentration of substrate: thus the pH optimum with 0·2% of carboxymethylcellulose was 6·3, whereas with 0·8% of substrate it was 5·6. As the enzyme was not saturated (see below), the pH optima could be expected to vary with substrate concentration (Dixon & Webb, 1958). Generally the optimum pH was found to lie between pH 5 and 6. Since more than one enzyme is possibly involved, the pH optimum will vary if defined in terms of reducing sugar liberated from a long-chain molecule. The amount of D-glucose produced from cellophan in the initial stages of hydrolysis ($\leqslant 2\%$) was found to be less than the total reducing material expressed as glucose, the difference varying with pH.

Effect of varying substrate concentration. Whereas cellobiase activity reached a maximum at a substrate concentration of 0·1–0·2% and then showed a fall in activity, the activity of carboxymethylcellulase was still increasing with substrate concentration when the solutions became too viscous to concentrate further (1·5%). Increasing concentration of cellophan up to a 3% (w/v) suspension in the incubation mixture, and cellulose dextrin up to 1%, likewise did not reach the maximum activity of the enzyme.

Relative rates of hydrolysis of substrates. The amounts of reducing sugar (expressed as glucose), produced by 0·2 ml. of enzyme from 0·8% of substrate in 2 hr. at pH 6·1, were 800, 125 and 10 μg. from carboxymethylcellulose, cellulose dextrin and cellophan respectively.

Extent of hydrolysis of substrate by prolonged enzyme action

Incubation mixtures containing 0·08% of carboxymethylcellulose and 0·8 ml. of enzyme were analysed at intervals; the reaction was found to be virtually complete after 3 hr., corresponding to 21% hydrolysis of substrate, assuming 0·5 sodium carboxymethyl unit per glucose residue. With 0·08% of cellulose dextrin, under similar conditions, a figure approaching 10% hydrolysis (5% measured specifically as glucose) was obtained.

Reaction of 0·8 ml. of enzyme with a 0·5% suspension of cellophan produced 16% hydrolysis in 7 days and the washed residue after re-incubation with more enzyme was hydrolysed a further 6%. Substantial inactivation of carboxymethylcellulase activity occurred after incubation for 7 days, amounting to about 80% for enzyme incubated alone and nearly 90% for that incubated in the presence of cellophan.

To establish whether a greater concentration of enzyme produced a substantial increase in hydrolysis of cellophan, a preparation concentrated fivefold by evaporation in a vacuum desiccator at 10° was added, along with buffer, in four daily portions, to 5 mg. of substrate, to constitute finally a volume of 1 ml. After incubation for 4 days total, 25% of the substrate had been hydrolysed. The carboxymethylcellulase activity of enzyme incubated alone was 50% of the original and that incubated with cellophan slightly less.

Dr C. A. Marsh kindly investigated the effect of intact rumen micro-organisms on 20 mg. quantities of cellophan and was able to demonstrate 95% solubilization of substrate, after 4 days. An interesting observation here is that the cellophan lost its glossy appearance and became opaque during digestion by the micro-organisms, but did not disintegrate to any considerable extent, whereas after digestion by cell-free extracts there was no difference in appearance but substantial disintegration into small pieces on gentle shaking.

Effect of gluconolactone on enzymic hydrolysis

It has previously been shown that cellobiase may be completely inhibited by gluconolactone, but that reducing-sugar production from carboxymethylcellulose is only partially inhibited under the same conditions (Festenstein, 1958*a*). Present studies with glucose oxidase to measure glucose specifically have shown that glucose production from both carboxymethylcellulose and cellulose dextrin is very largely inhibited ($\geqslant 85\%$) by 2·5 mM-lactone, whereas the overall reducing-sugar production is not inhibited to the same extent. The effect of gluconolactone on the enzymic hydrolysis of cellosaccharides is shown in Table 1; similar

Table 1. *Effect of* D-*glucono*-1 → 4-*lactone on enzymic hydrolysis of* mM-*cellosaccharides*

Solutions were incubated with enzyme for 45 min. at pH 6·1; in the range investigated cellobiose and cellotetraose were hydrolysed at a uniform rate.

Substrate	Concn. of lactone (mM)	Inhibition (%) Total reducing substances	Inhibition (%) Glucose
Cellobiose	0·5	—	99
Cellotriose		74	90
Cellotetraose		71	75
Cellotetraose	2·5	78	92
Cellopentaose		75	82

amounts of hydrolysis products were obtained for the substrates investigated, relative percentage hydrolyses (taking cellobiose as 1·0) for the series from cellobiose to cellopentaose being 1·0:2·4:1·4:1·1.

Effect of other inhibitors on carboxymethylcellulase

Halliwell (1957*a*) has shown that toluene (12 %, v/v), NaF (0·02 M) and thymol (87·5 % saturated solution) cause substantial inhibition of hydrolysis of cellulose powder by rumen micro-organisms; the effect of these substances was tested on reducing-sugar production from carboxymethylcellulose (0·4 %). It was found that toluene (0·2 ml./ml. of aqueous incubation mixture) had no effect, NaF (0·05 M) inhibited very slightly (6 %) and thymol (half-saturated) inhibited slightly (17 %); solutions without inhibitor indicated about 4 % hydrolysis of substrate.

Chromatographic analysis of hydrolysates

Cellophan. Determination of glucose, as well as total reducing substances, showed that after 0·5 % hydrolysis of 10 mg. of cellophan, 50 % of the reducing material appeared as glucose, the value rising to 84 % for 1 % hydrolysis and 96 % for 3 % hydrolysis, remaining in this region even when the limiting figure had been reached (experiments at pH 5). The products obtained from cellophan incubated with enzyme to give > 1 % hydrolysis were chromatographed and sprayed with aniline phosphate, which detected principally glucose as expected; there were also substances which gave pink colours with the reagent, R_G 0·11, 0·82 and 1·25, the latter spot coinciding with xylose (solvent butanol–pyridine–water, 6:4:3, by vol.). The $AgNO_3$ reagent of Trevelyan, Procter & Harrison (1950) detected, in addition, traces of substances with R_G 0·17 and 0·66 (cellotriose? and cellobiose, possibly) as well as an undifferentiated spot stretching from the origin to include cellotetraose (R_G 0·05).

Cellulose dextrin. Investigations after extended hydrolysis (10 %) showed that only half the reducing material consisted of glucose, although this was the major single product even after 2 % hydrolysis. Several substances appeared after chromatography, including some undifferentiated spots near the origin; specimen R_G values were 0·13, 0·20, 0·36, 0·48 (solvent butanol–pyridine–water, 6:4:3, by vol.) and 0·06, 0·13, 0·22, 0·34 (solvent butanol–pyridine–water, 6:2:3, by vol.). Corresponding values for cellotetraose, cellotriose and cellobiose were 0·05, 0·25, 0·64 and 0·04, 0·17, 0·47 respectively. The products were detected by both benzidine in trichloroacetic acid and $AgNO_3$, though the relative intensities of the spots differed; there was inadequate resolution in acetic acid–propan-2-ol–water (10:58·5:31·5, by vol.) (Hash & King, 1954).

Carboxymethylcellulose. Previous studies of hydrolysates obtained in the presence and absence of gluconolactone and analysed by the acetic acid-containing solvent showed undifferentiated substances up to R_G 0·2 with traces of substances with R_G 0·23, 0·58 and 0·74 in addition to cellobiose (R_G 0·80), the substance with R_G 0·23 appearing only in the presence of lactone (Festenstein, 1958*a*). On the basis of the data of Hash & King (1954), who used this particular solvent system and obtained R_G 0·76 for cellobiose, it was suggested that the substances with R_G 0·23 and 0·58 may have been cellopentaose and cellotriose respectively. However, subsequent studies with authentic cellopentaose and cellotriose gave R_G 0·03 and 0·35 respectively and the value obtained for cellotetraose was 0·09 (Hash & King claimed that a substance with R_G 0·35, obtained from hydrolysis of cellulose by *Myrothecium verrucaria* enzyme, was cellotetraose).

Although several other hydrolysates were examined, differentiated spots up to R_G 0·2 were not generally obtained and the substance with R_G 0·23 sometimes extended in position from R_G 0·20 to 0·35. It was possible, however, occasionally to identify substances with R_G 0·01 and 0·07, which were definitely increased in the presence of lactone and migrated with cellopentaose and cellotetraose in butanol–pyridine–water (6:4:3 and 6:2:3, by vol.), though in these solvents cellopentaose hardly moved from the origin; it was not possible to identify the original substance with R_G 0·23 after chromatography with butanol-containing solvents, but it seems likely that it behaved like cellotriose, which appeared in the same area as gluconolactone on the chromatograms.

DISCUSSION

The relative rates of hydrolysis of carboxymethylcellulose and cellophan resemble those obtained by Jermyn (1952) for *Aspergillus oryzae* enzyme and by Holden & Tracey (1950) for snail digestive juice, though in the latter case the enzyme preparation appears to be much more powerful than the rumen extracts, since 70 % hydrolysis of cellophan could be demonstrated in 19 hr.

The lower limiting hydrolysis of cellophan by the rumen preparation, about 25 %, may be due to exposure of resistant crystalline regions in the substrate rather than to the presence of an intrinsically resistant residue requiring a different mechanism for hydrolysis. The physical appearance of the residual cellophan was quite different according as hydrolysis had taken place by the rumen extracts or by the intact micro-organisms. It would seem that the influence of local enzyme concentration and also possibly inhibition by glucose should be further investigated.

Toluene, sodium fluoride and thymol have only small effects on reducing-sugar production from carboxymethylcellulose, yet Halliwell (1957*b*) found substantial inhibition of digestion of filter paper by intact rumen micro-organisms. Since these materials interfere with bacterial metabolism and cause glucose to accumulate, it is possible that continuous removal of glucose and its further metabolism are factors accelerating the initial stages of breakdown of filter paper and also cellophan by intact rumen micro-organisms; also the initial stage of breakdown may require energy, which is provided by the catabolism of glucose.

The effect of gluconolactone on hydrolysis of the lower cellosaccharides shows that inhibition is strongest towards the smallest molecule, cellobiose, and becomes progressively less effective as the chain length increases. However, inhibition of cellopentaose hydrolysis is still substantial, with 2·5 mM-lactone, particularly in respect of glucose production. The general finding of inhibition by lactone, along with the chromatographic studies of the hydrolysis of carboxymethylcellulose in the presence of lactone, suggests that small amounts of cellobiose, cellotriose and higher saccharides accumulate in the presence of lactone, but the dividing line is diffuse rather than sharp and several products accumulate rather than a single one.

The rate of hydrolysis of the cellosaccharides is not significantly increased with chain length, in accord with the finding of Grassmann, Zechmeister, Tóth & Stadler (1933) for *Aspergillus oryzae* enzyme, rather than that of Whitaker (1956) for *Myrothecium verrucaria* culture filtrates. Youatt (1958) has recently shown that a cellobiase preparation from *Stachybotrys atra* hydrolyses cellosaccharides from cellobiose to cellopentaose, and also a cellulose dextrin of mean chain length 11, at comparable rates, but has no action on carboxymethylcellulose. This particular cellobiase is evidently not inhibited by gluconolactone (M. A. Jermyn, personal communication).

Cellobiose was not detected in cellulose dextrin hydrolysates and only traces appeared in the cellophan hydrolysates; evidence for the higher cellosaccharides was only suggestive, emphasizing the powerful cellobiase activity of the rumen preparations.

SUMMARY

1. Specificity studies on cell-free extracts of cellulolytic enzymes from sheep rumen have been extended to cellophan and cellulose dextrin, which have been found to be hydrolysed at lower rates than carboxymethylcellulose, particularly cellophan.

2. D-Glucono-1 → 4-lactone has been shown to be a specific inhibitor of glucose production from carboxymethylcellulose, cellulose dextrin and also cellosaccharides from cellobiose to cellopentaose, producing at least 80 % inhibition at a concentration of 2·5 mM.

3. Inhibition of hydrolysis of mM-cellosaccharides in the series cellobiose–cellopentaose by 2·5 mM-gluconolactone has shown the hydrolysis of cellobiose to be completely (99 %) inhibited, the inhibition decreasing with chain length to 82 % for cellopentaose. The inhibition measured in terms of reducing sugar is generally less than that in terms of glucose.

4. Chromatographic analysis of carboxymethylcellulose hydrolysates with cellosaccharides as reference substances suggests that small amounts of cellobiose and possibly higher saccharides accumulate in the presence of gluconolactone; other substances, probably substituted derivatives, complicate the analysis.

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4. THE ABSORPTION OF 7-DEHYDROCHOLESTEROL IN THE RAT*

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It was shown previously that sterols undergoing absorption in the guinea-pig intestine become distributed uniformly among the cell fractions and are not confined to fatty globules (Glover & Green, 1957). The sterol composition of each cell fraction was found to be the same, indicating that an exchange and transfer of the sterols took place between them. It was suggested that this process played an important part in absorption and indicated that sterols were absorbed at the molecular level.

The present work was undertaken to see if a similar situation obtains in another species, the rat, which has been the main experimental animal used for lipid-absorption studies. A preliminary account of the work has been described (Glover & Stainer, 1957).

7-Dehydrocholesterol was selected for the test because the rat intestine, in contrast with that of the guinea pig, contained little (Glover, Glover & Morton, 1952) and its characteristic spectral absorption serves as an excellent label for tracing its movement across the cell.

EXPERIMENTAL

Materials. 7-Dehydrocholesterol was obtained from Peboc Ltd., Liverpool, and was recrystallized from methanol before use until $E^{1\%}_{1\,cm.}$ 281·8 mμ was 286–290.

Animals. Male rats in the weight range 140–265 g. were divided into groups of two having approximately the same mean weights. Four days before selection for experiment they were placed on a lipid-free diet of solvent-extracted food cubes (no. 4 diet, British Extracting Co. Ltd., Port Sunlight). Control groups (C1–C7) were fasted for 24 hr. and then killed for examination as described previously (Glover *et al.* 1952). Each animal in the test groups (D1–D8) was given a dose of 150 mg. of 7-dehydrocholesterol dispersed in 0·5 ml. of arachis oil and mixed into 10 g. of lipid-free diet. The animals were killed 5 hr. later for examination. In four experiments (D5–D8), however, each animal was given a similar dose 24 hr. before the final dose. The additional material was administered to ensure a reasonable level of absorption along the intestine at the time of examination.

Preparation of mucosal cell homogenate. The proximal half of the small intestine was removed from each animal and immediately flushed out several times with ice-cold 0·25 M-sucrose solution. The tissue was then cut into 4 in. lengths, opened to expose the mucosa and laid flat on top of large Petri dishes containing salt–ice mixture. The mucosal layer was scraped off gently with the back of a scalpel and transferred with five times its volume of 0·25 M sucrose solution into a pre-cooled Potter–Elvehjem-type homogenizer having a Teflon (Fluon) pestle. The mucosal cells were comminuted for 2 min. with the tube immersed in salt–ice mixture.

The volume of the homogenate was noted (about 25–30 ml.) and a portion, usually one tenth, taken for sterol analysis. The fractionation of the remainder was carried out in a refrigerated centrifuge at −5 to +5°. A modification of the procedure used by Glover & Green (1957) was introduced for the control groups C4–C7 and the dosed animals D1–D8. It was desired to obtain more concentrated groundplasm fractions for nitrogen assays and electrophoresis studies (results to be reported in another paper). Consequently, the homogenate in the centrifuge tubes was covered with a layer of light petroleum–ether (50:50, v/v) and subjected to 20 000 *g* for 90 min. The centripetally moving fatty particles collected in the organic solvent (F layer) and the supernatant groundplasm fraction (I) were then carefully separated; the latter was thus obtained without dilution with washings. The sedimented particulate material was recomminuted in the homogenizer with the same volume of 0·25 M-sucrose as before and then fractionated to yield the fractions: 'cell debris and nuclei', mitochondria, microsomes, groundplasm (II). Although the two supernatant fractions, groundplasm (I) and (II), were analysed separately, the results for sterol analysis have been combined for presentation. The above-mentioned microsome fraction is obtained

* Part 3: Glover & Green (1957).

at a relatively low *g* value and hence contains mainly the larger lipid-containing granules and not the smaller particles with a high nucleic acid content (Barnum & Huseby, 1948).

This modified procedure was advantageous in dealing with the mucosal homogenates from the dosed animals, which with their increased fat content tended to agglutinate, and not fractionate easily.

Extraction of lipids and sterol estimations. The lipids were extracted from each fraction after the denaturation of the proteins as previously described (Glover & Green, 1957). The total, 'slow-' and 'fast-reacting' sterols were assayed by the Moore–Baumann (1952) method. The latter group, having a double bond in the 7:8 position, are also referred to as Δ^7-stenols.

The 7-dehydrocholesterol was determined spectrographically (Glover *et al.* 1952). It should be mentioned here that for the purposes of applying the correction for irrelevant absorption, it is advisable that the standard curve on which the correction procedure is based should be obtained on the instrument used for the routine analysis, and modified correction points selected if necessary.

RESULTS

The distribution of the total sterols in the various cell fractions from the fasting and dosed animals is shown in Table 1. The variation in the mean values for the fasted groups C1–C3 compared with those for C4–C7 inclusive arises from the difference in fractionation technique. With C4–C7 the fatty layer was removed first by spinning the homogenate for 90 min. at 20 000 *g*. The redispersion of the sedimented particles probably breaks down the residual whole cells which had previously escaped rupture. Thus the microsomes and groundplasm fractions become larger at the expense of the 'cell debris and nuclei' fraction, which includes unbroken cells.

The mean value for the total sterol content of the mucosal homogenate from the dosed animals is approximately 3–7 mg. greater than that for the controls, but the percentage distribution of the total sterols among the various cell fractions remains much the same. This indicates that the sterol absorbed from the lumen becomes uniformly dispersed among the organelles and is not retained in the fatty globules which make up the F-layer fraction. The latter, in fact, from both the fasting and control animals, contains only 3 % of the total cell sterols.

The amount, however, by which each mucosal cell fraction increases its sterol content during absorption of 7-dehydrocholesterol is discussed in the next paper (Glover, Green & Stainer, 1959).

The contents of 'fast-reacting' Δ^7-stenols, lathosterol and 7-dehydrocholesterol, expressed as a percentage of the total sterols in each cell fraction, are given in Table 2.

Table 1. *Distribution of total sterols in the intestinal mucosal cells of fasting rats and animals dosed with 7-dehydrocholesterol*

There were two rats in each group. Dose: 150 mg. in experiments D1–D4 and 300 mg. in D5–D8. A modified fractionation procedure was used for control groups C4–C7 and for the dosed animals. Results for each fraction are expressed as a percentage of total sterols in the homogenate.

	C1–C3		C4–C7	
	Range	Mean ± S.D.	Range	Mean ± S.D.
	Control groups			
Total sterols in homogenate (mg.)	6·5–9·5	8·1	6·5–7·5	7·1
Cell fraction:				
Cell debris and nuclei	14·4–27·2	20·5 ± 6·4	6·2–15·1	9·6 ± 3·9
Mitochondria	11·1–15·4	13·2 ± 2·2	5·4–17·0	10·0 ± 5·1
Microsomes	24·4–36·9	31·3 ± 6·4	30·0–48·4	39·9 ± 8·1
Groundplasm	23·4–27·6	25·5 ± 3·0	27·1–34·4	31·9 ± 3·4
F layer	1·3–4·0	2·3 ± 1·5	0·8–5·4	2·8 ± 2·1
Recovery (%)	76–94	—	81–100	—
	D1–D4		D5–D8	
	Range	Mean ± S.D.	Range	Mean ± S.D.
	Dosed groups			
Total sterols in homogenate (mg.)	8·5–14·6	10·2	11·6–16·1	14·4
Cell fraction:				
Cell debris and nuclei	7·0–12·4	9·1 ± 2·9	6·4–10·2	8·7 ± 1·7
Mitochondria	7·2–22·5	17·2 ± 7·2	8·6–16·6	11·8 ± 3·4
Microsomes	31·7–38·9	36·2 ± 3·3	31·2–40·0	36·2 ± 3·7
Groundplasm	17·3–30·4	25·6 ± 5·9	22·4–43·5	30·7 ± 9·1
F layer	1·8–8·1	4·2 ± 2·8	1·8–2·5	2·1 ± 0·3
Recovery (%)	84–90	—	85–98	—

Table 2. *Percentage of Δ^7-stenols, 7-dehydrocholesterol and lathosterol in the total sterols of the cell fractions from the intestinal mucosa of fasting rats and animals dosed with 7-dehydrocholesterol*

Cell fraction	Total Δ^7-stenols (%)		7-Dehydrocholesterol (%)		Lathosterol (%) (by diff.)	
	Range	Mean ± S.D.	Range	Mean ± S.D.	Range	Mean ± S.D.
	Control animals (seven experiments)					
Homogenate	8·4–22·9	14·7 ± 5·4	2·9–4·3	3·6 ± 0·6	5·0–19·6	11·1 ± 5·4
Cell debris and nuclei	6·9–20·0	14·7 ± 4·9	2·1–5·3	3·5 ± 1·3	4·3–15·5	11·2 ± 4·8
Mitochondria	10·4–22·0	15·6 ± 4·3	1·9–6·9	4·4 ± 1·9	5·8–14·8	11·2 ± 3·9
Microsomes	5·5–16·7	11·7 ± 4·6	1·6–3·5	2·5 ± 0·7	3·9–14·4	9·2 ± 4·3
Groundplasm	6·5–22·4	11·1 ± 5·8	2·7–5·4	3·7 ± 0·9	1·1–19·4	7·4 ± 6·3
F layer	4·4–45·8	23·1 ± 17·1	0·8–7·5	4·6 ± 2·6	1·2–38·3	18·5 ± 15·0
	Dosed animals (eight experiments)					
Homogenate	51·7–68·4	62·7 ± 5·9	17·4–26·0	22·2 ± 2·6	34·3–46·2	40·5 ± 4·3
Cell debris and nuclei	47·1–74·4	62·8 ± 10·9	13·2–26·9	19·4 ± 4·6	20·2–58·2	43·4 ± 12·3
Mitochondria	57·8–83·5	71·0 ± 7·7	10·5–28·3	18·4 ± 5·9	15·0–56·5	52·6 ± 11·1
Microsomes	40·5–71·9	59·5 ± 10·0	20·4–34·2	25·4 ± 5·1	20·1–50·9	34·1 ± 12·0
Groundplasm	53·1–70·1	64·0 ± 5·8	12·7–26·9	19·0 ± 4·8	29·8–57·4	45·0 ± 8·7
F layer	55·1–84·7	69·8 ± 12·8	11·1–30·8	18·8 ± 7·8	36·2–69·8	51·0 ± 11·7

In the control groups, the 'fast-reacting' Δ^7-stenols make up 15% of the total, 7-dehydrocholesterol accounts for 3–4% and the remainder is regarded as being mainly lathosterol. These results are in agreement with those of Moore & Baumann (1952). In the dosed animals, the quantity of Δ^7-stenols (47%) is greater, as expected, but the increase in 7-dehydrochlolesterol (approx. 19%) accounts for only a small part of the total increment. Thus a considerable portion of the 7-dehydrocholesterol absorbed has apparently been reduced enzymically at the 5:6 double bond to form lathosterol. The content of the latter in each cell fraction is given by the difference between the values for total Δ^7-stenols and 7-dehydrocholesterol.

There was no significant increase in the cholesterol content of the intestinal mucosa of the dosed animals over that of the controls. Clearly, the different endogenous sterols must be rapidly exchanged and mixed between the cell components. The 7-dehydrocholesterol absorbed from the lumen of the intestine and its transformation products also enter into these exchange and transfer processes.

DISCUSSION

The above results for the distribution of total sterol across the intestinal mucosal cell of the rat are in general agreement with those obtained by Schotz, Rice & Alfin-Slater (1953) for rat liver, when account is taken of the higher *g* value used by these workers in obtaining the microsome fraction. They differ a little, however, from the previous results obtained in this Laboratory on the guinea pig (Glover & Green, 1957). The difference (a smaller 'cell debris and nuclei' fraction and larger groundplasm fraction) is probably due more to the modification of the technique mentioned above than to a species difference.

In keeping, however, with the results of most workers using mainly liver tissue, the microsome fraction contains the largest portion of the sterol content of the mucosal cell.

With regard to the 'fast-reacting' Δ^7-stenols, the total amount in the mucosal tissue of the fasting rat (10–20% of the total sterols) is very similar to that for the fasting guinea pig (Glover & Green, 1957). In the latter species, however, 7-dehydrocholesterol makes up half of this amount whereas in the rat it accounts for only a quarter. It is possible to change markedly the sterol composition of the tissue by prolonged feeding of a given sterol (Gould, 1955; Glover & Green, 1956), which displaces some endogenous material. The effect, if any, on cell function remains to be determined. However, soon after the administered sterol is withdrawn from the diet the characteristic sterol composition or equilibrium is restored.

The results in Table 2, showing that the distribution of the various sterols among the cell components tends to be uniform, confirm the previous findings of Glover & Green (1954, 1957) for the guinea pig. The fact that the newly formed lathosterol, which has increased from 11 to 40% of the total sterol in the dosed animals, is also evenly mixed shows that the exchange and transfer process between the lipoproteins of the various cell components is more rapid than the enzymic reduction of the 5:6 double bond of 7-dehydrocholesterol.

It is postulated that in the rat, as well as the guinea pig, the sterol-exchange and -displacement process in lipoproteins aids the transfer of the sterols across the mucosal cells and thus their absorption. The sterols finally transferred into the

lymph will therefore contain a mixture of dietary and endogenous sterols together with reduced or oxidized derivatives formed by the action of reductases (or cholesterol dehydrogenases) within the mucosa.

Recently Swell, Trout, Hopper, Field & Treadwell (1958) have also observed that dietary [4-^{14}C]cholesterol mixed with the endogenous sterols of the intestine of the rat during absorption. It should be noted, however, that this exchange and mixing process involves only the endogenous sterols of the mucosa and connective-tissue layers, and not those of the muscle layers, with which the dietary sterols scarcely come into contact. It was shown previously that the sterol compositions of these two neighbouring types of tissue are quite different and therefore do not take part in the above rapid-exchange system (Glover *et al.* 1952; Glover & Green, 1957).

SUMMARY

1. The distribution of total sterols, 7-dehydrocholesterol and lathosterol between the various cell fractions of intestinal mucosa of fasting rats and animals dosed with 7-dehydrocholesterol has been determined.

2. In both groups of animals, about 50% of the total sterols in the tissues was associated with the microsomes and mitochondria, 30% with the supernatant or groundplasm and 10% with the 'cell debris and nuclei' fraction. The mitochondrial fraction of the dosed rats contained more of the total sterol (15%) than the corresponding fraction from fasting animals (10%).

3. In agreement with previous work on the guinea pig, the various sterols were uniformly distributed among the components of the mucosal cells of both fasting and dosed groups, implying that the accumulated dietary sterol can exchange or mix with the endogenous sterols of the cell components.

4. It is also postulated that in the rat the uptake and exchange of sterols between the mucosal cell lipoprotein plays an important role in the absorption of sterols. The sterols in excess of the fasting level which are finally transferred to the lymph will, however, contain a mixture of the dietary and endogenous types displaced by the exchange process.

5. A large part of the 7-dehydrocholesterol absorbed from the intestine is also reduced within the mucosa to lathosterol.

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Sterol Metabolism

5. THE UPTAKE OF STEROLS BY ORGANELLES OF INTESTINAL MUCOSA AND THE SITE OF THEIR ESTERIFICATION DURING ABSORPTION

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The total sterols in the intestinal mucosa of the fasting guinea pig, previously maintained on a cholesterol-free diet, are invariably unesterified in the free alcohol form (Glover, Glover & Morton, 1952). On the other hand, it has been widely established since the work of Mueller (1916) that absorbed cholesterol appears in the lymph mainly as the ester, irrespective of the form in which it is administered. Cholesterol esters are hydrolysed mainly in the lumen of the intestine before absorption, but the position in the gut wall where resynthesis of esters commences has not yet been satisfactorily settled. Favarger & Metzger (1952) administered single doses of deuterated cholesterol oleate to rats, and found that the proportion of the labelled sterol in the free form was greater in the

mucosa than in the lumen of the intestine, implying that the action of the esterase is mainly hydrolytic in the mucosa. They then suggested that re-esterification of the free sterol probably occurred in the lymph.

It is necessary to establish, however, if esterification can occur to any appreciable extent within the mucosa during the absorption of cholesterol so that the role of cholesterol esterase in the latter process may be more clearly defined. The presence of cholesterol esterase within the mucosa means only that cholesterol esters might be formed from absorbed cholesterol if other conditions were satisfied. Such conditions could be an adequate supply of fatty acids or that the cholesterol should not be bound as a lipoprotein complex. Recent work on the absorption of cholesterol and 7-dehydrocholesterol in the guinea pig (Glover & Green, 1954, 1957) and the rat (Glover & Stainer, 1956, 1959) suggests that absorption of sterols is mediated via an exchange and transfer process between the lipoproteins of the mucosa. Under the conditions of these experiments, where absorption is occurring only over a limited period, the sterol may not then be esterified within the mucosa. The present work was planned to examine this point.

At the same time the measurement of sterol/nitrogen ratios for various mucosal cell fractions of fasting and dosed animals was undertaken to determine the extent of the uptake of absorbed sterols by each fraction during absorption.

EXPERIMENTAL

Materials

Recrystallized cholesterol (British Drug Houses Ltd., London) was purified further by forming the dibromide derivative and regenerating the free sterol as described by Schwenk & Werthessen (1952). Crystalline 7-dehydrocholesterol (obtained from Peboc Ltd., Liverpool) was freshly recrystallized from methanol as required.

Animals

Female guinea pigs (250–550 g.), divided into six pairs having approximately the same average weight (350 g.), were placed on a lipid-free diet (Glover *et al.* 1952) for 3 days before selection for experiment and then fasted for 24 hr. The control animals were examined at this stage. The test animals were given a dose of 300 mg. of cholesterol each in two stages: first, 150 mg. dispersed in 1 ml. of arachis oil was given mixed in approx 5 g. of lipid-free diet. After 24 hr. the remaining 150 mg. was given in the same way. The animals were killed 5½ hr. later. It was hoped that at this time the animals would be absorbing cholesterol along the greater part of the intestine.

Twelve groups of rats (140–265 g.) were prepared similarly. These animals were dosed with 7-dehydrocholesterol in two stages but each 150 mg. was mixed with 10 instead of 5 g. of lipid-free diet as used in guinea-pig experiments.

Methods

Cell-fractionation. The intestinal mucosal layer was scraped off the freshly removed intestine, placed in 25–30 ml. of 0·25 M-sucrose solution and comminuted for 90 sec. at 0° in a Potter–Elvehjem homogenizer fitted with a Teflon pestle. The cell fractions ['cell debris and nuclei', mitochondria, microsomes, supernatant groundplasm and F layer (fatty particles)] were prepared by high-speed centrifuging as previously described (Glover & Green, 1957).

A slight modification was introduced for the groups of rats used for determination of sterol/nitrogen ratios. The volume of sucrose added to the mucosa was only 10–15 ml. and the homogenate was centrifuged immediately at 20 000 *g* under a layer of light petroleum (b.p. 40–60°)–ether (50:50, v/v) at 5° for 90 min. Thus the F layer and supernatant, or groundplasm, fractions were obtained first, and the latter with a minimum amount of sucrose contaminant for the nitrogen assay. The pellet from the first high-speed centrifuging, containing all the particulate fractions, was then resuspended in 25–30 ml. of sucrose solution and fractionated by differential centrifuging as before. The supernatant from this fractionation labelled groundplasm (ii) was not assayed for nitrogen. Each fraction was divided into two equal parts, one of which was used for sterol assay and the other for nitrogen determination.

Determination of total, free and esterified sterols. The lipids were extracted from the various fractions as previously described (Glover & Green, 1957) and the total sterol content was determined on a suitable portion by the procedure described by Kenny (1952). It was considered better to look for small amounts of ester by a paper-chromatographic method than to estimate its presence in the filtrate from the precipitation of free sterol as the digitonide. A modification of the reversed-phase technique developed by Kodicek & Ashby (1954) worked well. The procedure was checked by carrying out some analyses on lipids from blood samples previously analysed by standard methods: the free cholesterol as digitonide (Sobel & Mayer, 1945) and total cholesterol by the methods of Abell, Levy, Brodie & Kendall (1952) and of Kenny (1952).

Paper-chromatographic procedure. Strips of Whatman no. 3 MM paper (1·5 in. × 10 in.) are immersed in a solution of liquid paraffin in light petroleum (5%, v/v), drained and dried at 60° for 10 min. The mobile phase is ethanol–water (95:5) mixture. The mixed lipid containing 1 mg. of total sterol is applied in chloroform solution from a micropipette (0·2 ml.) along a thin zone (0·5 cm. wide) about 4 cm. from one end of the strip. The paper is then mounted for ascending chromatography with this end just dipping in the mobile phase. The esterified cholesterol remains at the origin and the free material moves with R_F 0·8. The separation therefore takes place quickly and the paper may be examined about 1–2 hr. after commencing the chromatography, when the solvent front has moved 10–15 cm. An additional sample is usually run as a qualitative standard to check the position of the sterol zones, which may be located by spraying the dried paper with antimony trichloride reagent, and heating, as described by Kodicek & Ashby (1954). An alternative procedure is to cut the chromatogram into sections 2 cm. wide, which are folded and extracted with 3 ml. of chloroform in a test tube. The

paper is then lifted out and the Liebermann–Burchard test performed.

For the quantitative assay the two zones containing the different forms are cut out (up to 3 cm. wide) together with a blank zone taken above the solvent front. These pieces are folded and placed in a test tube (0·5 in. diam.) and covered with 3 ml. of chloroform. The tubes are shaken a few times at room temperature and the extract is transferred to a 10 ml. graduated tube fitted with a glass stopper. The papers are then washed twice with 3 ml. of ether and the bulked ether extracts carefully evaporated to dryness in test tubes (1 in. diam.). The respective residues are then transferred to the main extracts, not more than 2 ml. of chloroform being used for each. The final volume of each extract in the graduated tubes is made up to 5 ml., and the sterol content of the solution estimated spectrophotometrically at 410 mμ (Green, Lowe & Morton, 1955). The blank reading is deducted from the test and the amount of free or esterified sterol estimated from standard curves for the free and ester forms respectively.

The various results on blood-lipid samples are set out for comparison in Table 1. In a few cases, additional cholesterol or cholesterol ester was added to the blood lipids and this material was recovered quantitatively in the correct fraction.

Nitrogen determination. The various cell fractions used for nitrogen assay were made up to known volume with water and an appropriate portion was taken for analysis by the standard micro-Kjeldahl procedure with the Markham (1942) distillation apparatus. Each fraction was assayed in duplicate, and with each set of determinations duplicate ammonium sulphate standards and triplicate blanks were run.

Vitamin A absorption. One experiment with two guinea pigs was performed in the same manner with vitamin A alcohol in order to compare its absorption with that of the sterols. Each animal was given 22 mg. of vitamin A alcohol in 0·5 ml. of arachis oil. The free and combined vitamin A were separated from the extracted lipid of the intestine by column chromatography on alumina (Peter Spence and Sons Ltd., Widnes, Lancs.; grade 0) weakened by the addition of 5% of water (v/w) (Macqueen, 1948).

RESULTS

Occurrence of sterol esters in intestinal mucosal cells

When the mucosal cell fractions of four groups of fasting guinea pigs were examined for esterified sterols none could be detected. In the same number of experiments with animals dosed with cholesterol, sterol ester was found in the groundplasm fraction of only one group.

In rat intestinal mucosa, on the other hand, sterol esters were encountered more often but sporadically. In only two out of five groups of fasting rats examined were sterol esters detected. In both instances, esters were found in the groundplasm and F-layer fractions and in one group also in the microsome fraction. Similarly, esterified sterols were found in only three out of five groups of rats dosed with 7-dehydrocholesterol. Again, it was detected in the groundplasm (one group) and

Table 1. *Comparison of methods of estimating free and esterified cholesterol*

Results for free and esterified cholesterol separated by reversed-phase paper chromatography are compared with those obtained from the measurement of total cholesterol and the free alcohol as digitonide (Sobel & Mayer, 1945).

Blood-lipid sample no.	Free sterol		Ester		Total*	
	Digitonide (mg.)	Chromatography (mg.)	By difference (mg.)	Chromatography (mg.)	Direct method (mg.)	Chromatography (mg.)
1	0·160	0·160	0·390	0·390	0·550	0·550
2	—	0·175	—	0·375	—	0·550
2	0·155	0·140	0·395	0·400	0·550	0·540
3	0·275	0·280	0·635	0·605	0·910	0·885
4	0·145	0·135	0·255	0·290	0·400	0·425
	—	0·155	—	0·265	—	0·420
5	0·220	0·200	0·460	0·450	0·680	0·650
	—	0·205	—	0·428	—	0·633
6	0·268	0·258	0·250	0·247	0·518	0·505
7	0·425	0·409	0·483	0·476	0·908	0·885
	—	0·415	—	0·470	—	0·885
8	0·311	0·296	0·127	0·130	0·438	0·426
	—	0·315	—	0·135	—	0·450
9	0·285	0·300	0·850	0·830	1·135	1·130
	—	0·295	—	0·827	—	1·122
10	0·765	0·760	0·480	0·476	1·245	1·236
	—	0·778	—	0·463	—	1·241

* Estimated in samples 1–5 by the method of Abell, Levy, Brodie & Kendall (1952) and in samples 6–10 by the Kenny (1952) procedure.

F-layer (two groups) fractions. When sterol esters were detected, the amounts present were invariably quite small, less than 50 μg.

The greater tendency for esterified cholesterol to appear in the F layer and groundplasm of the rat is possibly due to the fact that the rat absorbs fat a little better than the guinea pig (Cook & Thomson, 1951), and hence the immediate concentration of fatty acids within the mucosa may be higher during the absorption of the cholesterol.

In the comparative experiment in which vitamin A alcohol was examined the absorption pattern is different. In sharp contrast with the sterols this experiment shows the ester form present in every cell fraction of the guinea pig (Table 2). Approximately 0·5% of the original dose was in the mucosa at the time of examination. This is consistent with the observations of Glover, Goodwin & Morton (1947), who could not detect vitamin A in the mucosa of the fasting rat, and of Gray & Cawley (1942), who showed that it became esterified on absorption. It appears that the mucosal cell fractions have the capacity to take up lipids in addition to those present in the fasting state just as vitamin A ester is taken up by liver-cell fractions (Schotz, Rice & Alfin-Slater, 1953).

Table 2. *Distribution of absorbed vitamin A in the cell fractions of the total intestinal mucosa from two guinea pigs examined 5·5 hr. after dosing*

Each animal was given 22 mg. of vitamin A alcohol in 0·5 ml. of arachis oil.

	Vitamin A		
	Total (μg.)	Ester (μg.)	Percentage as ester
Debris and nuclei	44	35	80
Mitochondria	46	22	48
Microsomes	45	35	78
Groundplasm	45	27	60
F layer	65	65	100
Whole mucosa (calc.)	245	184	75

Sterol/nitrogen ratios in mucosal cell fractions

Results for the total sterol/nitrogen ratios of the various mucosal cell fractions from fasting and dosed guinea pigs and rats are given in Table 3. The values for the corresponding fractions from the fasting animals of the two species are very similar. They are approximately double, however, the values for rat-liver fractions as calculated from the results of Spiro & McKibbin (1956). The generally higher ratios for the dosed animals indicate that the absorbed sterol is taken up by the lipoproteins of the organelles as well as simply exchanging with endogenous material (Glover & Green, 1957).

DISCUSSION

The present results show that cholesterol or 7-dehydrocholesterol esters are not formed to an appreciable extent in the mucosa of animals receiving a single dose. The fact that esters were detected only in trace amounts and sporadically implies that the esterification of the sterol is not essential for its passage across the outer-cell membrane or 'brush border'. Furthermore, the higher sterol/nitrogen ratios for the dosed animals compared with the controls indicate that the absorbed sterols have been taken up by the organelles. The implication is that although a readily available supply of fatty acids or neutral fat (from arachis oil in the dose) is also present in the cell, sterol absorbed in small amounts must be associated with protein or lipoprotein in a form which does not easily become esterified. In this connexion, Favarger & Metzger (1952) showed that a mucosal preparation in the presence of oleic acid readily esterified free cholesterol added in aqueous

Table 3. *Uptake of sterols by the various cell fractions of intestinal mucosa of the guinea pig and rat during the absorption of cholesterol and 7-dehydrocholesterol*

Two animals were used in each group and each was dosed with two 150 mg. portions of the sterol in 0·5 ml. of arachis oil, 24 hr. and 5½ hr. before examination.

		Total sterol (mg.)/nitrogen (mg.)			
		Guinea pig		Rat	
Cell fraction		Controls (3 expts.)	Dosed (3 expts.)	Controls (4 expts.)	Dosed (8 expts.)
Cell membranes and nuclei	Range	0·11–0·15	0·30; 0·39	0·19–0·23	0·26–0·94
	Mean (±S.D.)	0·13 (±0·02)	0·35	0·21 (±0·02)	0·52 (±0·25)
Mitochondria	Range	0·18–0·21	0·21–0·27	0·18–0·28	0·31–0·67
	Mean (±S.D.)	0·19 (±0·02)	0·23 (±0·03)	0·23 (±0·05)	0·45 (±0·14)
Microsomes	Range	0·28–0·33	0·34–0·45	0·34–0·40	0·42–0·62
	Mean (±S.D.)	0·31 (±0·03)	0·41 (±0·06)	0·35 (±0·03)	0·52 (±0·07)
Groundplasm	Range	0·02–0·03	0·11; 0·07	0·03–0·05	0·06–0·15
	Mean (±S.D.)	0·03 (±0·005)	0·09	0·05 (±0·02)	0·08 (±0·03)

dispersion but not free cholesterol added in the form of the lipoprotein complex existing in plasma. It is also interesting to contrast the behaviour of vitamin A with that of the two sterols. The greater proportion (75 %) of the former is readily esterified within the mucosal cell, although here a different enzyme may be involved. The experiment with vitamin A, however, suggests either that a much greater pool of fatty acids must be required to bring about the esterification of intestinal sterols or that the nature of the binding of the two substances to the cell proteins is quite different.

Cholesterol, like vitamin A, eventually appears in the chyle and in the chylomicra mainly in the esterified form (Daskalakis & Chaikoff, 1955; Page, Lewis & Plahl, 1953), but when labelled cholesterol was administered to rats radioactivity was first detected in the free fraction of lymph (Hellman, Rosenfeld, Gallagher, Adlersberg & Wang, 1953). Thus it appears that some cholesterol at least may enter the chyle in the free form. This is consistent with the work of Hernandez, Chaikoff, Dauben & Abraham (1954), who found that although *epi*-(3α-OH)cholesterol is not esterified by cholesterol esterase, it is readily absorbed into the lymph from the intestine although at only one-half of the rate characteristic of cholesterol. So, esterification of 3β-OH sterols probably takes place as they leave the mucosal cells and enter the connective tissue or in the lymph. As the proportion of ester in the quite rapidly flowing lymph is fairly constant, around 70 %, the former site is favoured. It is here that the incoming sterol will first tend to accumulate, and the excess, over and above that which can be held in a complex with protein, will then be exposed to the action of esterase. Esterification could then be regarded as ancillary to the absorption process in that it can accelerate its rate but is not apparently obligatory. The occasional occurrence of cholesterol esters in the F layer and microsome fraction of both species might be explained by assuming that some of the mucosal cells in the immediate region of optimum absorption had accumulated more cholesterol than could be accommodated as a lipoprotein complex involving the free hydroxyl group; the excess would then become esterified. An additional factor is the concentration of fatty acids within the mucosa, which, as mentioned previously, is likely to be greater in the rat. Cholesterol esters accumulate in the mucosal cells of the guinea pig when administration of the sterol is continued over a number of days (Green, 1955). There is also little doubt that the lymph channels beneath the mucosal layer will also contain sterol esters, but in general single doses of sterol do not yield any appreciable ester in the mucosa.

The manner by which the additional free or esterified sterols are held by the various organelles of the mucosa is not yet clear. From the sterol/nitrogen ratios it would appear that their lipoproteins can carry a variable quantity of sterols. Furthermore, it is possible to change the type of sterol associated with the organelles through exchange during prolonged feeding with a particular sterol (Glover & Green, 1956). However, it still remains to be determined if any marked change in the morphology or function of the mitochondria (or microsomes) is brought about by these qualitative and quantitative changes in sterol content.

SUMMARY

1. Sterol esters are absent from the intestinal mucosal cells of fasting guinea pigs and occur only occasionally in the groundplasm fraction of cells from animals dosed with cholesterol. In the rat, sterol esters were found sporadically in the mucosa of both fasting animals and animals dosed with 7-dehydrocholesterol, mainly in the groundplasm and F-layer fractions. The results imply that esterification is not directly involved in the absorption of sterols across the outer-cell membrane.

2. By contrast, a single dose of vitamin A is mainly (approximately 75 %) esterified during absorption across the intestinal mucosa of the guinea pig. The vitamin becomes associated with each cell fraction.

3. A comparison of the sterol/nitrogen ratios for the cell fractions of fasting animals with those of sterol-dosed animals indicates that additional sterol can be taken up by the organelles. The latter therefore appear to play an important part in sterol absorption.

4. It is postulated that in these two species esterification of single doses of cholesterol occurs mainly in the connective tissue before its entry into the lymph vessels, and that the action of esterase, although not obligatory for absorption, greatly accelerates the process.

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Biochemical Aspects of Bovine Ketosis

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Although studies on ketosis arising from diabetes, starvation and other causes have occupied a host of workers in the past, comparatively little is known of the biochemical aspects of bovine ketosis. Apart from the increased occurrence of ketone bodies in the blood and urine, bovine ketosis is characterized by abnormally low concentrations of blood glucose and liver glycogen (Shaw, 1955). It was decided to analyse the blood of diseased animals for the presence of abnormal quantities of metabolites, such as amino nitrogen, urea, ammonia and (in particular) members of the citric acid cycle. Of the 12 constituents estimated, which included cholesterol, in the serum of approximately seventy normal and diseased animals, the investigation of the levels of pyruvate, citrate, α-oxoglutarate and succinate proved the most fruitful. The highly significant abnormalities in the levels of these four components in the serum of ketosed animals represent the main result of this work.

Only one team of workers was found to have reported studies on the levels of pyruvate and α-oxoglutarate in the literature. In these Carlström, Myrbäck, Holmin & Larsson (1939) suggested that in bovine ketosis the blood levels of the two keto acids were above normal, though the opposite view was taken with regard to pyruvate in later work (Carlström, 1944, 1950).

One of the tasks undertaken was the elaboration of specific methods for the estimation of quantities of serum constituents as low as 1–2 μg. Another point considered was the decomposition of the blood components during the transport and clotting period in which they were exposed to ambient temperature. This problem has been studied extensively in preceding work (Bach & Hibbitt, 1958), when storage conditions were found which would ensure the stability of the serum constituents until their estimation was possible.

From the results of the present work it was concluded that in bovine ketosis an interference may take place with certain reactions of the Krebs cycle, and further that these disturbances may assist in the explanation of the other biochemical symptoms of the disease. Though no significant differences were found between the serum levels of the nitrogenous components and of cholesterol in normal and ketosed animals, it is thought that the record of results for these constituents in normal and abnormal serum may fill a gap in the literature.

METHODS

Animals. All the blood samples from normal and ketosed animals were obtained from cows of the Jersey breed. Some of the normal samples were collected from the Veterinary Field Station of the University of Bristol and the remainder of the normal samples as well as all samples from animals suffering from ketosis were collected from Jersey Island.

Collection of samples and analytical methods. The procedure for the collection of blood samples was identical with the one described in previous work (Bach & Hibbitt, 1958), as were the methods used for the estimation of amino nitrogen, urea, ammonia, ketone bodies, cholesterol and glucose. In view of the findings in the above-mentioned work about the lability of the various serum components, care was taken that these were estimated within that period of time after collection during which the components had previously been found to be stable at ambient temperature (Bach & Hibbitt, 1958).

Estimation of pyruvate and α-oxoglutarate. Hydrazones of these compounds were prepared according to standard methods, the modification described in previous work

(Bach & Hibbitt, 1958) being used. The modifications were necessary to avoid the interference of acetoacetic acid in the serum of ketosed animals. The hydrazones were separated by paper chromatography, and were eluted and colorimetrically estimated.

Estimation of citric acid. The pentabromoacetone method was adapted for serum and modifications were elaborated to overcome the interference of ketone bodies and of acid contaminants with the colour reaction (Bach & Hibbitt, 1958).

Estimation of succinic acid

A new method was elaborated which was sensitive enough for the estimation of quantities as low as 1 μg.

Principle. The succinic acid is extracted from deproteinized serum with ether. After evaporation of the ether, the residue is dissolved in phosphate buffer and the enzymic reduction of cytochrome *c* by succinic acid in the presence and absence of malonate is determined spectrophotometrically.

Procedure. A portion (10 ml.) of serum was deproteinized in a centrifuge tube by adding 50 ml. of tungstic acid reagent [10% sodium tungstate–water–2/3 N-sulphuric acid (1:3:1, by vol.)]. After standing for 10 min. the protein was separated from the supernatant by centrifuging. The succinate was extracted with 20–30 ml. of ether from a known volume (approximately 50 ml.) of the supernatant for 16 hr. in a Kutscher–Steudel extractor. After the extraction the ether was evaporated under reduced pressure and any traces of moisture in the residue were removed by desiccation *in vacuo*. The residue was dissolved in 2 ml. of 0·3 M-phosphate buffer (NaH_2PO_4–NaOH), pH 7·6.

To two 1 cm. cells (capacity 4 ml.) of a Unicam SP. 500 spectrophotometer were added 0·5 ml. of the phosphate buffer, 0·2 ml. of sodium cyanide (0·03 M) and 0·5 ml. of a well-washed Keilin & Hartree (1947) succinoxidase preparation in which the final sediment had been suspended in 0·1 M-phosphate buffer (NaH_2PO_4–NaOH), pH 7·3, containing 0·8 M-sucrose. Further additions to both cells were: 0·5 ml. of cytochrome *c* (0·214 mM) and 1·1 ml. of water. The contents of the cells were gently mixed and a preliminary reading was taken at 550 mμ. With accurate pipetting and thorough mixing a zero reading can be obtained, though in a few cases this initial reading (R_1) differed slightly from zero, usually by less than 0·001 *E*. After the initial reading a portion (0·4 ml.) of the 2 ml. of serum extract was added to one cell and an equal volume of water to the other. Readings were taken at 3–5 min. intervals until there was no further reduction of the cytochrome *c*, when the final reading (R_2) was taken. With an active enzyme preparation the readings became constant after 10–30 min.; enzyme preparations taking more than 45 min. were discarded. This procedure was then repeated in the presence of malonate, which was added to both cells in a final concentration of 0·1 M before the initial reading. In this way a second pair of readings (R_3 and R_4) were obtained. The value $(R_2 - R_1) - (R_4 - R_3)$, which ranged from 0·010 to 0·035, was considered to be the extinction caused through the reduction of cytochrome *c* by the succinate present in the serum extract. The succinic acid level was calculated from a calibration graph, known amounts of succinate in aqueous solution being used. The same cytochrome *c* preparation was used for the calibration as was used for the estimation of the serum extract. Recovery experiments for succinate are shown in Table 1. In one set (*A*) a known amount was added to untreated serum, then extracted from the serum and estimated. In another set (*B*) the succinate solution was added to the serum extract and then estimated; in a third set (*C*) aqueous succinate solutions were estimated. It is evident that the losses occurring in set *A* were mainly due to incomplete extraction of the succinic acid from the serum.

RESULTS

Glucose and ketone bodies. Table 2 shows the main biochemical characteristics of bovine ketosis, namely a considerable depression of the glucose

Table 1. *Recovery experiments with succinate*

(*A*) Serum (10 ml.) containing added amounts of succinate was deproteinized with the tungstic acid reagent (50 ml.) and 50 ml. of the protein-free solution was extracted with ether. The residue of the extract was dissolved in 0·3 M-phosphate buffer, pH 7·6 (2·0 ml.), and a sample (0·4 ml.) used for spectrophotometric determination of succinate, as described in Methods. (*B*) Succinate was added to an ether extract of normal serum. (*C*) Aqueous solutions of succinate were used instead of the serum extract.

(*A*) Succinate in 10 ml. of serum			(*B*) Succinate in 0·4 ml of ether extract			(*C*) Succinate in 0·4 ml. of water	
Added (μg.)	Found (μg.)	Recovery (%)	Added (μg.)	Found (μg.)	Recovery (%)	Added (μg.)	$E_{550\ m\mu}$ (duplicate experiments)
0·0	6·6	—	0·0	1·1	—	1·0	0·012 0·010
6·0	10·8	70	1·0	1·9	80	2·0	0·022 0·023
12·0	14·4	65	2·0	2·9	90	3·0	0·036 0·034
18·0	20·4	77	0·0	1·2	—	4·0	0·048 0·049
24·0	23·4	70	3·0	4·0	93	—	—
—	—	—	4·0	4·9	92	—	—

level and a high ketone-body level in the serum of approximately 30 ketosed animals, as compared with the levels of 36 normal ones. The glucose levels of both normal and diseased animals compare well with the findings of other workers where the normal values ranged from 41 to 92 mg./100 ml. (MacDonald, Krueger & Bogart, 1956) and the ketonaemic values from 15·3 to 42·4 mg./100 ml. (Sampson, Gonzaga & Hayden, 1933). Total ketone bodies in normal animals amount usually to no more than 11 mg./100 ml. (Thin & Robertson, 1953), as compared with 30–106 mg./100 ml. in bovine ketosis (Table 2). The bulk of the ketone bodies in diseased animals were found to consist of acetone and β-hydroxybutyric acid; smaller quantities were present as acetoacetic acid.

It was tempting to investigate whether or not the two pathological phenomena were interconnected. Fig. 1 demonstrates an inverse relationship, so that the severity of the disease can be assessed by either of the two criteria. The position of the line in this figure was calculated as that best fitting the scatter of points. The correlation coefficient was found to be significant at a 5 % level.

Nitrogenous components and cholesterol. As far as can be ascertained from the estimation of concentrations of amino nitrogen, ammonia and urea in the serum of normal and ketosed animals, there appears to be no influence of the pathological condition on the metabolism of protein (Table 3). Moreover, except for the normal urea levels, the values obtained showed considerable consistency, not only for the normal but also for the ketosed animals. Though the slight depression of urea nitrogen observed in ketosis as compared with the normal values was not significant, a lower urea level might have been expected in the diseased animals, in view of their reduced food intake. The range of normal urea nitrogen values (6–22 mg./100 ml.) was similar to that given by MacDonald *et al.* 1956 (8·8–24·2 mg./100 ml.).

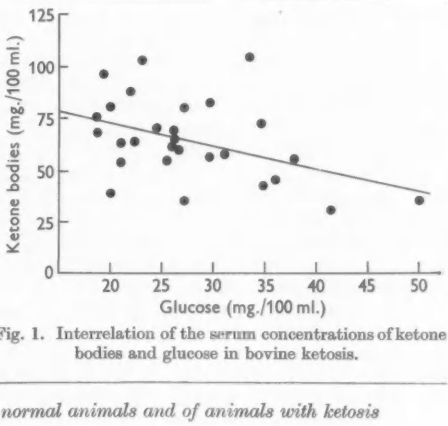

Fig. 1. Interrelation of the serum concentrations of ketone bodies and glucose in bovine ketosis.

Table 2. *Glucose and ketone bodies in serum of normal animals and of animals with ketosis*

	Normal		Pathological	
	No. of cases	Mean (mg./100 ml.)	No. of cases	Mean (mg./100 ml.)
Glucose	36	65·87 (40·0–93·0)	33	28·18 (18·6–40·0)
Acetone	—	—	30	34·1 (15·0–56·4)
Acetoacetic acid (as acetone)	—	—	—	6·21 (2·1–12·2)
β-Hydroxybutyric acid (as acetone)	—	—	29	24·7† (12·2–43·8)
Total ketone bodies (as acetone)	20*	6·1* (2·9–10·9)	29	65·6 (30·6–106·0)

* Thin & Robertson (1953).
† Uncorrected for the presence of propan-2-ol (Thin & Robertson, 1952).

Table 3. *Nitrogenous constituents and 'total' cholesterol in serum of normal animals and of animals with ketosis*

	Normal		Pathological	
	No. of cases	Mean ± S.E.M. (mg./100 ml.)	No. of cases	Mean ± S.E.M. (mg./100 ml.)
Amino nitrogen	33	5·08 ± 0·05	14	5·28 ± 0·08
Ammonia nitrogen	28	0·36 ± 0·04	14	0·47 ± 0·09
Urea nitrogen	35	13·52 ± 0·77	13	11·68 ± 0·70
Cholesterol	26	205·5 ± 8·52	14	199·5 ± 7·43

The ammonia levels in the serum of the normal and ketosed ruminants investigated were slightly higher than those reported in the literature for man. The increase may be due to the predominance of bacterial fermentation in the digestion of ruminants; it could also be argued that a part of the ammonia was formed in the serum during the clotting time by a fluoride-insensitive process.

No influence of the pathological condition was observed on the concentration of amino nitrogen and of cholesterol.

Pyruvate, α-oxoglutarate, citrate and succinate. Table 4 shows the serum concentrations of these members of the citric acid cycle in normal and ketosed animals. The differences in these levels between the two groups of animals amounted to several hundred per cent, and are highly significant. Moreover, the individual results showed a surprisingly high degree of consistency in spite of the complexity of the analytical procedure for such small quantities. The pyruvate in ketosed animals was more than 300% above normal and this phenomenon was paralleled by a strong depression in the amount of citrate. The results for concentrations of α-oxoglutarate and succinate were analogous; it was found that in ketosed animals the former was greatly increased and, from the few experiments carried out, that this increase was accompanied by a depression of the level of succinate. Because of the close metabolic relationship between pyruvate and citrate on the one hand and between α-oxoglutarate and succinate on the other, an interference with these metabolic reactions in the citric acid cycle was suspected; this will be discussed below. The blood level of pyruvate in normal cows, as estimated by older and less specific methods, was given by Carlström *et al.* (1939) as 0·4–0·6 mg./100 ml., and that for ketosed animals as 1·0–1·6 mg./100 ml. However, in later work Carlström (1944, 1950) reported that the ketonaemic levels were lower than the normal ones. A blood concentration of α-oxoglutarate in bovine ketosis as high as 8 mg./100 ml. was claimed by Carlström *et al.* (1939), though this was estimated by a less specific gravimetric method; Carlström (1944, 1950) also stated that the levels of citrate in the diseased animals were generally below normal.

Attempts to correlate the degree of abnormality in the concentrations of the four members of the citric acid cycle with the degree of severity of the disease, as assessed by the abnormal levels of ketone bodies and of glucose, were unsuccessful.

DISCUSSION

Inasmuch as the amounts of metabolites in the blood can reflect the nature of tissue metabolism, the results of the present investigations may throw some light on the biochemical aspects of bovine ketosis.

The accumulation of pyruvate and the corresponding depression of citrate in the serum of ketosed animals could be interpreted as an interference with the conversion of pyruvate into citrate via acetyl-coenzyme A. This phenomenon was paralleled by an accumulation of α-oxoglutarate and a depression of succinate, signifying a possible inhibition of the conversion of the former compound into the latter. Since the mechanism of these reactions is very similar and since the reactions require identical cofactors, there is reason to believe than an interference with the latter may be at the root of the disturbance in the Krebs cycle. Two factors which participate in the two reactions are of particular interest: coenzyme A and thiamine pyrophosphate. The fact that B_1 avitaminosis interferes with the oxidative utilization of pyruvic acid may have been the basis for the therapeutic experiments of Carlström (1950) and of Shaw (1946); neither, however, could find any beneficial effect from administration of the vitamin to animals suffering from bovine acetonaemia.

More evidence is available for the view that a deficiency in coenzyme A may be partly responsible for the observed abnormal concentrations of members of the Krebs cycle. From the work of Olson & Kaplan (1948), it was shown that a greatly reduced turnover of pyruvate in liver slices could be obtained from animals deficient in pantothenic acid and in coenzyme A. More support for the above view was obtained through the work of Peeters, Oyaert, Bouckaert & Haeck (1958), who reported that the acetylating capacity in ketosed animals was lowered as compared with normal

Table 4. *Tricarboxylic acid-cycle intermediates in serum of normal animals and of animals with ketosis*

	Normal		Pathological		
	No. of cases	Mean ± S.E.M. (mg./100 ml.)	No. of cases	Mean ± S.E.M. (mg./100 ml.)	% Normal
Pyruvate	28	0·55 ± 0·02	30	1·73 ± 0·26 (<0·001)*	314
Citrate	29	4·41 ± 0·09	33	0·89 ± 0·06 (<0·001)	20
α-Oxoglutarate	30	0·19 ± 0·01	30	0·60 ± 0·06 (<0·001)	316
Succinate	14	0·11 ± 0·007	5	0·06 ± 0·007 (<0·01)	55

* The figures in parentheses are probabilities calculated by the *t* test (Fisher, 1938).

animals. Certain clinical findings also support the view of a coenzyme A deficiency in ketonaemia. The animals are susceptible to the disease exclusively in the post-parturition period, which coincides with that of the highest milk and, in particular, fat production, i.e. when the synthesis of great quantities of butter fat demands excessive amounts of acetyl-coenzyme A. In experiments with guinea pigs it has been shown that the amount of coenzyme A in the mammary gland rises steeply in the period of lactation after parturition (Ringler, Baker & Nelson, 1954), and Read & Moore (1956) reported that injections of prolactin which induce lactation also increase the coenzyme A within an hour after injection. It may well be therefore that ketosis occurs as a consequence of the inability of the animal to meet the extra demand for coenzyme A. The demand for the latter is enforced through the fact that the main dietary source of fat in the ruminant is acetate, which has to be activated by coenzyme A for further utilization (Folley & French, 1950; Balmain, Folley & Glascock, 1954). According to Ringler *et al.* (1954), the changes in the concentration of coenzyme A follow the general trend of milk production, and it has been observed by one of us (K.G.H.) that ketosed cattle which maintain milk production at a high level are more resistant to therapy (consisting of intravenous glucose injections) than those in which lactation has rapidly decreased.

A deficiency of coenzyme A could be caused by a lack of adenosine triphosphate, since this is required in several stages in the biosynthesis of the coenzyme (Johnson, 1955). Administration of a mixture of creatine and adenosine triphosphate in conjunction with glucose has been reported to relieve the symptoms of ketosis immediately in many cases (Carlström, 1944, 1950). These observations were confirmed by Olsson (1945), who reported the successful use of an adenosine triphosphate preparation supplemented by glucose and creatine.

A severe hypoglycaemia is one of the striking characteristics of bovine ketosis, and it has been shown in this work that a direct relationship exists between these two symptoms (Fig. 1). The low blood sugar seems to be caused by a disturbance in the mechanism of carbohydrate synthesis which involves adenosine triphosphate, whereas the utilization of glucose remains unaffected (Holmes, 1951). An intereference with the synthetic glucose from pyruvate could thus be a contributing factor to the observed accumulation of pyruvate in the blood of ketosed animals. In agreement with this view, treatment of the disease was in the past mainly confined to attempts to increase the blood sugar by injections of glucose (Shaw, Powell & White, 1942) and of glucose-forming metabolites such as propionate (Schultz, 1952; Hoflund, Nordström & Hallgren, 1955), lactate (Seekles, 1951) and glycerol (Johnson, 1954). Administration of such components has met with success after prolonged treatment and it may thus be conceivable that through excessive doses of metabolites, the pace of the Krebs cycle is forced (Krebs, 1957) and the synthesis of adenosine triphosphate and glucose enhanced. Peeters *et al.* (1958), on the grounds of their acetylating experiments described above, came to strikingly similar conclusions.

SUMMARY

1. The sera of approximately 70 Jersey cows, half of them suffering from bovine ketosis, were analysed for their content of twelve selected blood constituents. These included four members of the citric acid cycle (pyruvate, citrate, α-oxoglutarate and succinate).

2. Known micro-methods were adapted for estimating small amounts of the above compounds and a new method for the estimation of succinic acid was elaborated.

3. A relationship was established between the two principal biochemical characteristics of the disease, i.e. the high concentration of serum ketone bodies and the very low concentration of glucose, so that either phenomenon can be used to assess the severity of the disease.

4. No significant differences between normal and diseased animals were found for amino-nitrogen, urea, ammonia and cholesterol.

5. In all pathological cases the pyruvate and α-oxoglutarate in serum were several hundred per cent above normal and this was paralleled by a strong depression of the amounts of citrate and of succinate.

6. It was concluded that in bovine ketosis there may exist an interference with the citric acid cycle, notably with those reactions in which pyruvate and α-oxoglutarate are converted into citrate and succinate respectively. From the known similarity of the mechanism of the two reactions a deficiency in cofactors common to both was suspected, such as coenzyme A and adenosine triphosphate; the compatibility of such a deficiency with certain symptoms in ketonaemia is discussed.

It is a pleasure to express our gratitude to Mr C. L. Gruchy and Mr G. McCallum, St. Helier, Jersey, as well as to Professor A. Messervy and Mr H. Pearson, University of Bristol, for their valuable help in providing us with blood samples. We also wish to thank Professor D. Keilin, F.R.S., Cambridge University, for advice on the estimation of succinate. We are indebted to the Agricultural Research Council for financial support for this work and in particular for a personal grant to one of us (K.G.H.).

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Unsaponifiable Constituents of Liver; Ubiquinone and Substance SC in Various Species

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The determination of substances present in very small amounts in the unsaponifiable fraction of liver lipids is made difficult by the fact that complete hydrolysis requires the tissue or lipid to be exposed to strong alkali on the boiling-water bath. Substances such as vitamins K and E are alkali-labile and indeed may be destroyed entirely. Cain & Morton (1955) found that when vitamin A was a relatively plentiful component of the liver, chromatographic and other methods of separation were often vitiated by the presence in the unsaponifiable fraction of vitamin A-decomposition products.

The substance SA of Festenstein, Heaton, Lowe & Morton (1955) and Cain & Morton (1955) was also encountered by Lowe, Morton & Harrison (1953) and by Heaton, Lowe & Morton (1955, 1957). Because of its widespread occurrence SA has been designated ubiquinone (Morton, Wilson, Lowe & Leat, 1957).

A second minor constituent of liver unsaponifiable material designated substance SC, was found in very small amounts in normal rat liver (Heaton *et al.* 1955) and larger amounts of both substance SC and ubiquinone were found in liver from vitamin A-deficient rats.

The main object of the present investigation was to determine whether ubiquinone and substance SC occur in the normal livers of various species.

Festenstein *et al.* (1955) found ubiquinone to be present in various tissues of pig, horse and rat, including normal rat liver. They showed that it was not a saponification artifact, since the same amount was present in the total lipid as in the unsaponifiable material from the tissue. Saponification of the extracted lipid, however, destroyed it. Thus since it is labile to alkali, ubiquinone is evidently protected in some way during the alkali digestion of the tissue. Ward & Moore (1955) confirmed the occurrence of an alkali-labile substance with the absorption band at 272 mμ of SA in the unsaponifiable material from vitamin A-deficient rat liver. They also observed similar spectra in liver fractions (*a*) from rats cured of vitamin A deficiency, (*b*) from sexually immature rats and (*c*) from normal pigs and normal guinea pigs. Cain & Morton (1955), in an extensive investigation of horse-liver unsaponifiable matter, found ubiquinone, usually accompanied by cholesta-3:5-dien-7-one. Although both substances show absorption peaks near 270 mμ, ubiquinone can readily be distinguished from the dienone. The latter is stable in alkaline solution and its spectrum in concentrated sulphuric acid has a characteristic peak at 356mμ; ubiquinone-

containing fractions, on the other hand, show no maximum at 356 mμ in concentrated sulphuric acid, but usually have a band with $\lambda_{max.}$ at 315 mμ (Kantiengar, Lowe, Morton & Pitt, 1955).

EXPERIMENTAL

Materials

Livers from healthy oxen, sheep and pigs were obtained from the Stanley Abattoir, Liverpool, and were always quite fresh. Cat livers were obtained from animals killed by the R.S.P.C.A. at the request of the owners. Dog livers were obtained from the R.S.P.C.A. or the Liverpool University Veterinary Hospital. Guinea-pig livers were obtained from animals kept in the Departmental Animal House for several months on a diet of cubes (Animal Diet no. 4, British Extracting Co., Bromborough, Cheshire) and fresh cabbage. Horse liver was obtained from Lasco Ltd., Liverpool, very soon after slaughter. The livers of 50 mice were obtained from Burroughs Wellcome and Co., London, and were examined as one lot; a similar batch of 50 livers was obtained from The Distillers Co. Ltd. (Speke). Livers from a number of pigs suspected of being vitamin A deficient were obtained from the Veterinary Investigation Centre, Liverpool, through the courtesy of Mr N. H. Brooksbank. The livers of three pullets were obtained from J. Bibby and Sons Ltd., Hann's Hall Farm, Willaston, Cheshire. Rabbit livers came from laboratory animals on a cube and cabbage diet. Human liver was provided by the Department of Pathology, University of Liverpool, through the courtesy of Professor H. L. Sheehan.

Methods

Saponification. If the liver could not be prepared very soon after reaching the laboratory it was stored at −20°, but this was usually not necessary. The tissue was cut into small pieces and refluxed on a water bath for 45–60 min. with a mixture of 60% (w/v) potassium hydroxide (0·5 ml./g. of tissue) and ethanol (1–2 ml./g. of tissue) containing pyrogallol (0·25%, w/v) which was added just before mixing. After cooling, water (2 vol.) was added and the solution extracted with five or six portions of ether (previously redistilled over reduced iron). The extract was washed, dried over Na_2SO_4, filtered through sintered glass and the solvent removed in a stream of nitrogen. The last traces of solvent were removed under reduced pressure in a 25 ml. flask on the water bath.

Chromatography. This was carried out on alumina (P. Spence and Co., Widnes) partially deactivated with water to Brockman Grade 3 (Williams, 1948). The column was washed with light petroleum (100–200 ml.), b.p. 40–60°, before use. The unsaponifiable matter was poured on to the column in light petroleum solution and the chromatogram was developed, at first with the pure solvent and then with mixed solvents consisting of light petroleum (b.p. 40–60°) containing increasing percentages of diethyl ether. The more polar compounds were finally eluted with ether and in some cases ethanol. All fractions were evaporated to dryness under nitrogen and weighed before spectroscopic examination in *cyclo*hexane. They were stored at −20° in the dry state in small conical flasks.

Spectroscopy. The weakly adsorbed material (mainly hydrocarbon) eluted from the alumina column by light petroleum showed an inflexion at 260 mμ (in *cyclo*hexane) but was otherwise free from selective absorption. α-Tocopherol and 7-dehydrocholesterol were determined by means of their well-known absorption spectra. Ubiquinone shows (in *cyclo*hexane) $\lambda_{max.}$ 272 mμ ($E^{1\%}_{1cm.}$ taken as 180 in calculating μg./g.) with an inflexion at 330 mμ and a weak band at 410 mμ. Substance SC (in *cyclo*hexane) shows two peaks, at 275 and 330 mμ respectively, with marked inflexions at 233 and 283 mμ (Fig. 1). Since the molecular extinction coefficients of SC are not known, the SC content of a preparation is expressed in arbitrary units xy, where $E^{1\%}_{1cm.}$ 275 mμ is measured (x) and the weight of the fraction is given in mg. (y).

Substance SC may occur generally in healthy liver but in most species the amount is so very small that no chromatographic fraction shows the characteristic absorption spectrum unambiguously. It is here reported as not detected (n.d.) unless the full spectrum was obtained, even though some fractions may have contained traces of substance SC but exhibited distorted spectra.

Preliminary separation of unsaponifiable fraction. Many samples of mammalian or avian liver contain up to 3000 i.u. of vitamin A/g., and this can with advantage be largely eliminated before undertaking chromatography. Cholesterol is readily crystallized from methanol and vitamin A remains in solution; ubiquinone and substance SC tend to accompany the cholesterol (Fig. 2). The first crop of cholesterol obtained in this way from a sheep-liver unsaponifiable fraction showed $\lambda_{max.}$ 275 mμ due mainly to ubiquinone.

A methanolic solution of dog-liver unsaponifiable matter was cooled in solid carbon dioxide and the methanol-insoluble fraction was quickly filtered off. The filtrate was warmed and cholesterol (0·2 g.) was added and dissolved. The solution was again cooled strongly and filtered. The

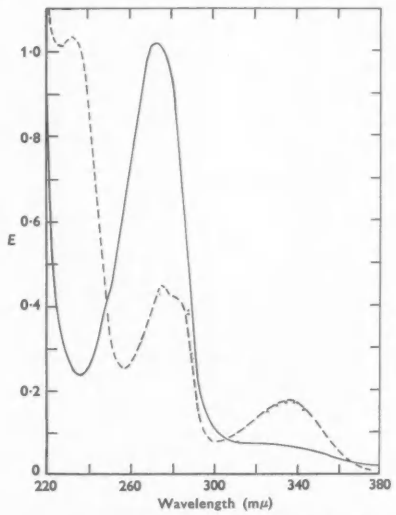

Fig. 1. ——, Absorption curve of ubiquinone in *cyclo*hexane; 1 cm., 0·006%. – – –, Absorption curve of substance SC in *cyclo*hexane; 1 cm., 0·0054%.

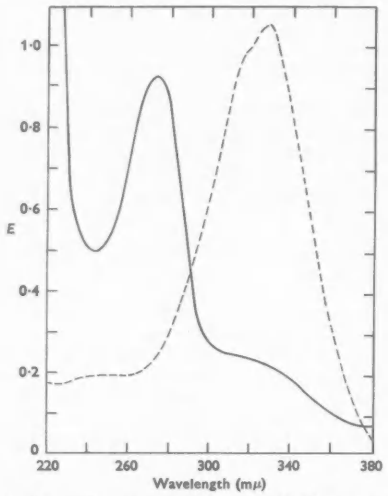

Fig. 2. Absorption curves of unsaponifiable matter of sheep liver. – – –, Methanol-soluble fraction in *cyclo*hexane (1 cm., 0·00176%); ——, methanol-insoluble fraction in *cyclo*hexane (1 cm., 0·617%).

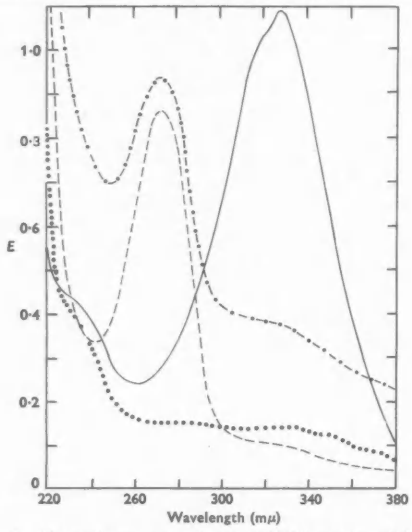

Fig. 3. Absorption curves of unsaponifiable matter of dog liver. 1 (——), Methanol-soluble fraction in *cyclo*hexane (1 cm., 0·0172%); 2 (– – –), methanol-insoluble fraction in *cyclo*hexane (1 cm., 0·307%); 3 (–·–·–), sterol obtained after adding cholesterol to 1 and recrystallizing from methanol; insoluble fraction in *cyclo*hexane (1 cm., 0·48%); 4 (. . . .), cholesterol added to mother liquor from 3 and crystallized. Methanol-insoluble portion in *cyclo*hexane (1 cm., 0·270%).

second crop of cholesterol crystals carried down an additional amount of ubiquinone. Addition of further cholesterol (0·2 g.) did not result in any further appreciable yield of material absorbing at 270–275 mμ (Fig. 3).

This procedure allowed most of the vitamin A to be separated from the mixture of cholesterol and ubiquinone. A careful test on a sample of unsaponifiable matter from normal pig liver, containing only a moderate amount of vitamin A, showed that the yield of ubiquinone obtained by chromatography of the whole unsaponifiable fraction was about 30 μg./g.; of this about 22 μg./g. was readily carried down by cholesterol. On the alumina column, ubiquinone is readily separable from cholesterol, but substance SC and 7-dehydrocholesterol tend to be eluted with cholesterol.

Acetylation of sterol fraction and rechromatography. The main sterol fraction eluted by 20% (v/v) ether in light petroleum can with advantage be acetylated (acetic anhydride and pyridine) and rechromatographed. Under these circumstances cholesteryl acetate, vitamin A acetate and 7-dehydrocholesteryl acetate can all be eluted by 1% (v/v) ether in light petroleum and any small amounts of more polar products can be eluted later with more polar mixtures of the two solvents.

RESULTS

The findings for the different species are first described and then summarized in Table 6.

Cat. The two cats studied gave liver unsaponifiable fractions exhibiting strong vitamin A absorption. Most of the vitamin A remained in solution after crystallization of cholesterol from methanol as described earlier. Cholesterol was added to the mother liquor and a fresh crop of crystals obtained. Table 1 shows that carotene and lycopene were detected along with 7-dehydrocholesterol, ubiquinone and two other entities, SB and SF (Morton & Fahmy, 1959). Rechromatography of the acetylated sterol fraction failed to reveal the presence in it of any substance SC.

Dog. Ubiquinone was present in fractions eluted by 4% (v/v) ether in light petroleum and 6% (v/v) ether in light petroleum. The main sterol fraction from dog 1 was acetylated and rechromatographed. The portion (0·7 mg.) eluted by 8% ether in light petroleum showed an inflexion at 227 mμ, a peak at 275 mμ ($E^{1\%}_{1\,cm.}$ 11·0) with inflexions at 282 and 315 mμ, all consistent with the presence of a trace of substance SC. The liver of the other dog did not give a detectable amount of SC.

Guinea pig. Four livers (103 g.) gave 0·311 g. of unsaponifiable matter showing maxima at 273, 282 ($E^{1\%}_{1\,cm.}$ 3·8 and 5·1 respectively) and 328 mμ. The vitamin A content was quite low (26 i.u./g. of liver), and it was therefore convenient to chromatograph the whole unsaponifiable fraction. The ubiquinone fraction was contaminated with a substance showing $\lambda_{max.}$ 297 mμ which turned out to be α-tocopherol. The next fraction also showed the tocopherol spectrum superimposed on that of

substance SC. The sterol fraction contained 7-dehydrocholesterol. After the sterol had been eluted, ether removed SF ($\lambda_{max.}$ 236, 282 mμ; $E^{1\%}_{1cm.}$ 48·0 and 36·3). Ethanol eluted an appreciable amount of xanthophyllic pigment. Rechromatography of the acetylated sterols confirmed the presence of 7-dehydrocholesterol and the absence of substance SC from the sterol fraction.

Horse. The two horse livers were low in vitamin A. Ubiquinone was present in both but neither substance SC nor SF could be detected unambiguously.

Mouse. The two specimens of mouse liver differed widely in vitamin A content. Nevertheless, ubiquinone was present in both at about the same rather high proportion (Table 2). The separation (Table 2) was clear, partly because of the absence of complicating carotenoids. Substance SC was not separated but SF was probably present in small amount.

Ox. Three samples were examined; the ubiquinone content was variable but substance SC was not detected although a distorted SC spectrum was suspected in one fraction.

Table 1. *Chromatography of methanol-insoluble portion of cat-liver unsaponifiable fraction*

Wt. of liver 129 g.; crude unsaponifiable matter 0·892 g.; cholesterol added to unsaponifiable matter 0·200 g. Methanol insoluble material 0·585 g.; a 200 mg. portion of this was chromatographed. Alumina column 20 g. E, Ether; P, light petroleum.

(*a*)

Fraction no.	Eluent (ml.)	Wt. (mg.)	Absorption spectra in *cyclo*hexane: $\lambda_{max.}$ (mμ)	$E^{1\%}_{1cm.}$	
1	200 P + 50 (1%) E/P	3·5	230	67·5	
			260	31·6	
			333	17·3	Mainly β-carotene
			432	42·8	
			451	53·1	
			480	43·7	
2	200 (2%) E/P	1·2	260	21·1	
			450	6·5	
			Max. 472	6·7	
			495	4·2, lycopene	
3	200 (4%) E/P	0·9	Max. 272	81·7, ubiquinone	
			330	14·4	
			362	9·3	
			Max. 445	9·6	
			Max. 472	10·1, lycopene	
			Max. 505	6·9	
4	200 (6%) E/P	8·4	Max. 274	3·2	SB?
			325	0·8	
5	100 (8%) E/P	1·7	No selective absorption, contains cholesterol		
6	700 (20%) E/P	181·8	257	0·68	
			265	0·70, 7-dehydrocholesterol	
			275	0·71	
			285	0·62	
			Max. 325	0·50, vitamin A	
7	200 (E)	1·3	Max. 287	19·0, ? SF	

(*b*) Fraction 6: acetylated and rechromatographed (0·1975 g. was placed on 20 g. column)

Fraction no.	Eluent (ml.)	Wt. (mg.)	Absorption spectrum in *cyclo*hexane: $\lambda_{max.}$ (mμ)	$E^{1\%}_{1cm.}$
1	400 (1%) E/P	191·4	7-Dehydrocholesterol and vitamin A acetate	
2	300 (4%) E/P	1·1	235	0·81
			270	0·40
3	300 (8%) E/P	0·4	235	4·86
			275	2·43
4	200 E	Trace	233	?
			270	—

Table 2. *Chromatography of mouse-liver unsaponifiable fraction*

Wt. of liver 88 g. (50 mice); unsaponifiable matter 0·277 g.; vitamin A, 10 i.u./g. of liver; unsaponifiable matter (0·258 g.) chromatographed on 20 g. of alumina. A second batch of mouse liver (from another colony) contained 151 i.u. of vitamin A/g., but the ubiquinone content was nearly the same (see Table 6). E, Ether: P, light petroleum.

Fraction no.	Eluent (ml.)	Wt. (mg.)	Absorption spectrum in *cyclo*hexane $\lambda_{max.}$ (mμ)	$E^{1\%}_{1cm.}$	
1	200, P	7·3	257	11·1	
			325	1·6	
2	200 (2%) E/P	0·2	280	51·5	
3	300 (4%) E/P	4·2	Max. 273	140·4	Ubiquinone
			320	14·4	
4	200 (6%) E/P	3·1	Min. 248	13·5, mainly ubiquinone	
			Max. 276	24·9	
			320	3·4	
5	100 (8%) E/P	1·8	Max. 276	7·9, SB	
			310	4·6	
6	800 (20%) E/P	161	235	1·4	
			287	1·3	
			320	1·9	
			Max. 327	2·0, vitamin A	
7	200 E	0·3	232	192	SF
			252	148	
			Max. 280	141	
			292	119	

Table 3. *Constituents of the unsaponifiable fractions of livers of pigs which died at 4–5 months of age*

—, Not measured. Substance SC was detected in trace amounts in samples 1, 2 and 4.

Sample no.	Wt. of liver	Unsaponifiable matter as percentage of liver weight	Total sterol (mg./g. of liver)	Vitamin A (i.u./g. of liver)	$\lambda_{max.}$ (mμ)	$E^{1\%}_{1cm.}$	Ubiquinone (μg./g. of liver)	7-Dehydrocholesterol (μg./g. of liver)
1	376	0·40	2·5	2·2	272	3·9	34·6	Not seen
2	173	0·41	2·3	4·2	272	3·4	46·8	Not seen
3	95	0·44	—	3·8	273	4·6	—	—
4	240	0·37	2·2	None	272	3·6	19·3	0·5

Pig. Three lots of liver from healthy pigs were examined. The vitamin A contents varied from 6 to 150 i.u./g. of liver but the ubiquinone varied only from 9 to 30 μg./g. Substance SB was present but SC could not be detected. Four samples were studied of liver from pigs which had died at the age of 4–5 months with symptoms of inco-ordination of the hind legs and increased excitability (Table 3). Although the ubiquinone content was rather high it was not at all clear that in pig liver this substance or SC increases in vitamin A deficiency. Substance SC was detected in small amounts and SF was present in the ethanol eluate from the alumina.

Pullet. The liver of the normal hen is a source of substance SC. Table 4 illustrates the chromatography of the methanol-insoluble unsaponifiable fraction. Fraction 5 (Table 4) was acetylated and rechromatographed. Fig. 4 illustrates the absorption spectrum of the 8% of ether in light petroleum eluate, which agrees with that of substance SC.

Rabbit. Two livers (104 g.) were bulked and the vitamin A content was determined (617 i.u./g.); cholesterol (0·2 g.) was added to the total unsaponifiable material in methanol. The crystallized cholesterol carried down ubiquinone but little vitamin A. The complete SC spectrum was not seen, but a trace of SC may have been present.

Sheep. The first two of the specimens studied (Table 6) were early ones, before the methanol crystallization with added cholesterol was adopted, and neither ubiquinone nor substance SC could be detected in the presence of vitamin A. In sheep 3 and 4, normal amounts of ubiquinone and minute traces of SC were detected.

Man. Table 5 records the results of examination of three human livers. Ubiquinone was present in

Table 4. *Chromatography of methanol-insoluble fraction of pullet-liver unsaponifiable matter*

Wt. of liver 124 g.; unsaponifiable matter 0·57 g.; vitamin A 950 i.u./g. of liver; cholesterol (0·2 g.) was added to 0·56 g. of unsaponifiable material dissolved in methanol and cooled. The crystalline product (0·55 g.) showed $\lambda_{max.}$ 273 mμ; $E^{1\%}_{1\,cm.}$ 3·4; 0·2 g. of this was chromatographed. E, Ether; P, light petroleum.

Fraction no.	Eluent (ml.)	Wt. (mg.)	Absorption spectrum in *cyclo*hexane: $\lambda_{max.}$ (mμ)	$E^{1\%}_{1\,cm.}$
1	50 (1%) E/P	—	Max. 260	19
			Max. 331	12
	+150 (2%) E/P	1·2	454	26, carotene
			482	22
2	300 (4%) E/P	1·9	Max. 272	150, ubiquinone
			320	14
3	200 (6%) E/P	3·0	Max. 272	26, ubiquinone
			320	2·8
4	100 (8%) E/P	1·1	Max. 274	73, SC?
			320	18·7
5	1000 (20%) E/P	176	Max. 274	0·41 } SC
			280	0·40 }
			320	0·31
			Max. 328	0·32, vitamin A
6	200 E	0·4	230	40
			270	26

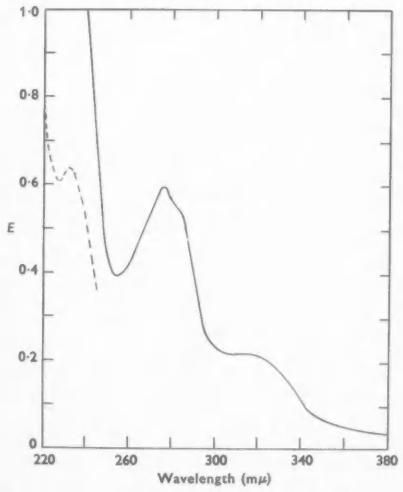

Fig. 4. Absorption curves in *cyclo*hexane of unsaponifiable fraction containing substance SC acetate from hen liver. This fraction was obtained by rechromatography of a fraction containing cholesterol and substance SC which had been acetylated to facilitate separation. ——, 0·0267%; – – –, 0·0134% (both measured in 1 cm. cells).

all, but substances SB, SC and SF could not be detected. Table 6 illustrates another case.

Table 6 recalls the main findings and Table 7 shows how widely the vitamin A content can vary compared with that of ubiquinone.

DISCUSSION

The unsaponifiable material of liver dissolved in methanol and strongly cooled deposits crystals of cholesterol, accompanied by ubiquinone, substance SC and 7-dehydrocholesterol. Vitamin A remains in the mother liquor and by adding further cholesterol and again crystallizing, additional amounts of trace constituents can be carried down. Chromatography then allows ubiquinone to be separated but SC remains with the sterol. If the sterol is acetylated, SC can be separated by chromatography from cholesteryl acetate. These procedures may lead to estimates erring on the low side because of losses occurring in the saponification. The use of pyrogallol in the hydrolysis greatly reduces such losses.

In the chromatographic separations 2–4% of ether in light petroleum eluted (well ahead of the cholesterol) a small amount of yellow oily material. This fraction was rich in ubiquinone. It was followed, with 4–6% of ether in light petroleum, by a substance in which $\lambda_{max.}$ was shifted from 272 to 274 mμ (solvent *cyclo*hexane) and the inflexion at 330 mμ was more marked than for ubiquinone. This substance, called SB for convenience, either possesses very low intensity of absorption or is in these experiments much diluted with congeners transparent to ultraviolet light. Less strongly adsorbed than ubiquinone is a hydrocarbon fraction eluted by petrol or 1% of ether in light petroleum. This almost always contains a substance showing an inflexion near 233 mμ and a small maximum or inflexion near 260 mμ.

Table 5. *Constituents of the unsaponifiable fraction from human livers*

Carotene and lycopene were present in traces in all three livers; tocopherol was found only in the third and substance SC was not detected in any of the three. These three specimens were examined by Dr L. Mervyn.

Sex	Age (years)	Cause of death	Wt. of liver (g.)	Percentage of unsaponifiable material	Sterol (mg./g. of liver)	Vitamin A (i.u./g.)	Ubiquinone (μg./g. of liver)
F.	72	Internal haemorrhage	120	0·74	4·8	2000	53
F.	20	Acute nephritis	110	0·68	3·2	240	56
M.	43	Uncertain	150	0·49	2·7	132	75

Table 6. *Unsaponifiable constituents of liver*

n.d., Not detected; d, detected; a, carotene; b, lycopene; c, xanthophylls; e, 7-dehydrocholesterol.

Species	Sterol (mg./g. of liver)	Vitamin A (i.u./g. of liver)	Ubiquinone (μg./g.)	Substance SC	Other compounds detected
Cat (i)	2·4	380	9	n.d.	a, b, e
(ii)	2·6	2320	13	n.d.	a, b, e
Dog (i)	1·3	104	20	d.	e
(ii)	1·4	61	3	n.d.	—
Guinea pig	2·0	26	11·6	?	c, e
Horse (i)	1·2	46	12	n.d.	e
(ii)	1·4	21	6	n.d.	a, e
Man	2·1	2000	12·7	n.d.	a, b
Mouse (i)	1·9	151	45	n.d.	e
(ii)	1·9	10	41	n.d.	e
Ox (i)	1·7	215	35	n.d.	a
(ii)	1·9	87	11·5	n.d.	a, b, e
	1·7	380	3	?	a, e
Pig (i)	2·4	6	9	n.d.	a
(ii)	1·7	15	24	n.d.	e
(iii)	3·1	160	30	n.d.	—
Pullet (i)	4·5	950	108	0·8 unit/g.	a, e
(ii)	2·4	1060	46	0·4	a, e
Rabbit	1·9	617	12·7	?	e
Sheep (i)	3·7	960	—	—	a
(ii)	3·1	540	—	—	a
(iii)	2·2	1830	15	d.	a, e
(iv)	3·8	1270	33	d.	a, e

Table 7. *Comparison of liver content of ubiquinone and vitamin A*

	Vitamin A (i.u./g.)	Ubiquinone (μg./g.)
Cat	382	8·5
	2320	12·5
Pig*	15	24·5
	160	29·8
Mouse	10	41
	151	44

* See also Table 3.

Substance SC, eluted by 20% of ether in light petroleum ($\lambda_{max.}$ 275, 330 mμ, inflexions 233, 283 mμ in *cyclo*hexane) was unmistakably observed in dog, sheep and pullet liver. It was not definitely detected in pig and other livers even if the vitamin A content was less than 10 i.u./g. of liver. In the rat, the SC content rises significantly in vitamin A deficiency (Heaton *et al.* 1955).

SF ($\lambda_{max.}$ 282 mμ) is occasionally found in liver unsaponifiable matter. It is very strongly held on alumina. This substance has been shown (Morton & Fahmy, 1959) to be 3-formylindole or a compound with the same absorption characteristics.

SUMMARY

1. Liver tissue saponified in the presence of pyrogallol yields unsaponifiable matter of which ubiquinone is a normal constituent.

2. The cholesterol which crystallizes from a methanolic solution of the unsaponifiable matter carries with it ubiquinone and substance SC. Addition of cholesterol to the mother liquor leads

to a second crop of crystals carrying more of the above. Vitamin A remains in the methanol-soluble fraction.

3. Chromatography on alumina allows the separation of ubiquinone from the cholesterol.

4. Acetylation of the sterol fraction followed by chromatography allows the separation of substance SC.

5. Ubiquinone has been found in normal liver of ten mammalian species in amounts from 10 to 40 μg./g. Pullet liver was richer. Substance SC was not often detected and then only in very small quantities.

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A Study of 'Unsaponifiable' Extracts Obtained from Hydrolysates of Pancreas and Spleen

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The primary object of the present work was to extend to pancreas and spleen a survey of quantitatively minor constituents of tissue unsaponifiable fractions. In particular it was desired to ascertain whether ubiquinone (SA) (Festenstein, Heaton, Lowe & Morton, 1955; Heaton, Lowe & Morton, 1955, 1957; Morton, Wilson Lowe & Leat, 1957) and α-tocopherol were present. In the course of the work a strongly adsorbed constituent was encountered and designated substance 'SF' until it was eventually identified as indole-3-aldehyde (3-formylindole) or a substance giving rise to 3-formylindole. The question arises whether this formylindole was an artifact derived from tissue proteins or some more specific precursor. Reference is made in the Discussion to the occurrence of 3-formylindole in products of biological interest.

The results of investigating pancreas and spleen from healthy animals made it desirable to study human tissue in a preliminary way. Ubiquinone and 3-formylindole have been obtained from human pancreas, and both substances, together with α-tocopherol, were found in human spleen. Unlike the cholesterol obtained from ox, sheep and pig spleen, which contained 7-dehydrocholesterol, the sterol from human spleen did not contain enough to be detected.

EXPERIMENTAL

Materials. Fresh pancreas and spleen from ox, sheep and pig were obtained from the Stanley Abattoir, Liverpool. The tissues were freed from adhering fat. In some experiments the lymph glands embedded in pancreas were excized and studied separately. Sheep pancreas was studied in some detail. The organ weighed from 36 to 53 g. (average of seven, 43 g.). Each contained oval pink glands, some small and some as big as a large bean. The glands varied in number from three to twelve (average eight) per pancreas. We have not found any references in the literature to these glands in sheep nor have our veterinary colleagues.

Human pancreas and spleen were obtained from the Department of Pathology, University of Liverpool, through the courtesy of Professor H. L. Sheehan. The tissue was obtained within about 12 hr. of death. It was at once cut into small pieces and saponified or left under ethanol at −20° overnight. Details of the three human spleens studied are: (i) Male, 41 years. Glioblastoma. Weight of spleen 99·9 g., 'unsaponifiable material' 0·506 g. (ii) Male, 55 years. Carcinoma of right suprarenal, metastases to liver, Cushing syndrome, severe hypokalaemia and hypocalcaemia. Weight of spleen, 121 g., 'unsaponifiable material' 0·395 g. (iii) Male, 74 years. Rheumatoid arthritis. Weight of spleen, 190 g., 'unsaponifiable material' 0·993 g.

Saponification. The following procedure (compare Tosic & Moore, 1945) tends to conserve tocopherols and ubiquinone. The tissue is cut into small pieces and weighed.

Pyrogallol (1 g./80 g. of tissue) is dissolved in ethanol (1 ml./2 g. of tissue) and added to the tissue. The mixture is quickly raised to the boiling point on a water bath. Potassium hydroxide solution (60%, 0·5 ml./g. of tissue) and more ethanol (1 ml./2 g. of tissue) are then added. Heating is continued for 20–45 min. After cooling, water (3 vol.) is added and the solution extracted with freshly redistilled ether, or occasionally with light petroleum. The bulked extracts are washed, dried and the solvent is removed. The unsaponifiable matter may be dissolved in hot methanol and cooled. The cholesterol which crystallizes is filtered off.

Chromatography. Alumina (grade O, P. Spence and Co., Widnes) was washed with acid, dried and partially deactivated to Brockman grade III (Williams, 1948).

Solvents. Light petroleum (b.p. 40–60°) was dried over sodium wire and distilled. Ether was usually dried similarly and distilled over reduced iron immediately before use.

Efficacy of pyrogallol in the saponification process. The effectiveness of pyrogallol as an antioxidant for known compounds such as vitamin K_1 and α-tocopherol was tested. It was reasonable to assume that experimental conditions allowing their recovery after refluxing with alkali would also favour conservation of ubiquinone and α-tocopherylquinone.

When menadione (2-methyl-1:4-naphthoquinone) was added to fatty material subjected to saponification it was not recovered; it had in fact formed a violet compound. The 2:3-disubstituted compound vitamin K_1 did not behave in this way. Provided that the time of saponification was not too long and that pyrogallol was added, both vitamin K_1 and α-tocopherol could be recovered to the extent of 85–90% after chromatography on acid-washed alumina.

Separation of ubiquinone and α-tocopherol. These two substances are eluted from acid-washed alumina together. If the fraction (freed from solvent) is treated with methanol at $-15°$ or $-25°$ the α-tocopherol is readily soluble. Under favourable conditions a very clean separation can be achieved.

RESULTS

Pancreas

Ox pancreas. Five batches of tissue (2·16 kg. in all) were used. In a typical experiment 343 g. of tissue gave 0·87 g. of 'unsaponifiable material' from which 0·35 g. of cholesterol was removed, leaving 0·52 g. to be chromatographed. Light petroleum eluted a red oil containing a little carotene. Saturated hydrocarbons (50 mg.) and cholesterol (257 mg.) were the main products. Ubiquinone and α-tocopherol were eluted by 2–4% (v/v) of ether in light petroleum in small fractions (2·5 mg. in all) coloured with carotenoids. Ether eluted an orange oil (4·8 mg.), which spectroscopically showed the presence of substance SF ($\lambda_{max.}$ 282 mμ, $E^{1\%}_{1\,cm.}$ 52) and xanthophylls. The recrystallized sterol showed no selective absorption.

Sheep pancreas. Three samples were studied, carotene, ubiquinone α-tocopherol and substance SF being found in each. The fractions eluted with 4 and 6% of ether in light petroleum were combined, evaporated to dryness under nitrogen and cooled to $-25°$ for 20 min. Methanol (5 ml.) was similarly cooled, poured on to the cold fraction and kept at $-25°$ for 1 hr. The supernatant liquid was decanted and the process repeated (Table 1).

At this stage it was decided to study the lipid as such. Sheep pancreas (259 g.) was homogenized once with ethanol and twice with ether. The combined extracts were washed, dried and the solvent was removed to give 4·1 g. of lipid. A portion of this was chromatographed as lipid and another portion was saponified and the unsaponifiable matter was chromatographed. The extracted tissue was hydrolysed in the presence of pyrogallol. The lipid chromatographed on alumina was eluted to the extent of 40% by light petroleum but total recovery was low (48·4%) because phospholipids were retained. It was not possible even by further chromatography of fractions to concentrate ubiquinone or α-tocopherol successfully. When the

Table 1. *Methanol separation of ubiquinone and α-tocopherol at* $-25°$

	$\lambda_{max.}$ (mμ)	$E^{1\%}_{1\,cm.}$	Wt. (mg.)
Initial product	280	80	12
First methanol-soluble extract	290	54·6	1·6
Second methanol-soluble extract	285–290	33·6	0·6
Third methanol-soluble extract	291	70·9	0·5
Methanol-insoluble	273	14·0	6·8

Table 2. *Comparison of pancreatic tissue with embedded lymph-gland tissue constituents of unsaponifiable fraction*

	Sheep pancreas		Ox pancreas	
	Pancreatic tissue	Glands	Pancreatic tissue	Glands
Unsaponifiable (%)	0·33–0·37	0·31–0·47	0·41	0·41
α-Tocopherol (mg./100 g.)	0·17	0·05	Not found	Not found
Ubiquinone (mg./100 g.)	0·4–0·6	0·8–1·4	0·88	1·6
Sterol* (g./100 g.)	0·13–2·0	0·15–0·27	0·17	0·29
Formylindole (μg./g.)	0·7–2·9	?–2·4	1·0–1·2	?–2·1

* 7-Dehydrocholesterol not found.

lipid (2·36 g.) was saponified in the presence of 0·2 g. of pyrogallol with boiling for 10 min. only, 0·2 g. of unsaponifiable matter was obtained. This gave a fraction (1·7 mg.), eluted by 4% of ether in light petroleum, containing both ubiquinone and α-tocopherol, and 6% of ether in light petroleum gave a fraction (3·3 mg.) showing the spectrum of α-tocopherol only. Cholesterol (105 mg.) was eluted by 20% of ether in light petroleum. The unsaponifiable matter from the extracted residue (190 mg.) showed the presence of ubiquinone, α-tocopherol, cholesterol (137 mg.) and substance SF (Table 2).

Pig pancreas. Five small batches of pig pancreas were prepared for analytical purposes and two large batches (17·7 kg.) for preparative purposes. The extracts contained very little carotenoid but ubiquinone, SF and other substances were present. Thus one unsaponifiable fraction (0·77 g.) yielded 14·4 mg. of ubiquinone of 85% purity directly (4–6% of ether in light petroleum eluates), 341 mg. of sterol and 16·4 mg. of a fraction containing substance SF.

Larger-scale preparations were found to contain up to 25% of ethyl palmitate formed by ester exchange. This is easily removed in the first stage of chromatography. In one experiment 3·83 g. of unsaponifiable matter yielded two fractions containing ubiquinone (51 and 22 mg.; $E^{1\%}_{1\,cm.}$ 117 and 51 respectively). Another fraction contained a congener of m.p. 43°. Ether eluted a fraction containing substance SF.

In brief, ubiquinone was found to the extent of 1–2 mg./100 g. in ox, sheep and pig pancreas. α-Tocopherol (1·6 mg./100 g.) was found only in sheep pancreas. Substance SF was obtained from the three species. The lymph glands also contained ubiquinone and substance SF.

Fat adhering to pancreas was examined separately. Ubiquinone was detected in trace amount in the fat attached to pig pancreas but not in that of sheep or ox. Tocopherol was not found. The fat adhering to ox pancreas yielded unsaponifiable matter which on chromatography revealed the presence of a very small amount of material with $\lambda_{max.}$ 266–267 mμ (*cyclo*hexane) eluted by 5% of ether in light petroleum and a trace of material with $\lambda_{max.}$ 261–262 mμ eluted by 6–7% of ether in light petroleum.

Spleen

Calf spleen. The unsaponifiable fraction contained traces of anhydrovitamin A, β-carotene, ubiquinone and 7-dehydrocholesterol (see Lovern, Morton & Ireland, 1939). Small amounts of substance SF were found in the ether eluate.

Ox spleen. This was similar but somewhat richer in ubiquinone (Table 3). The recrystallized sterol also contained some 7-dehydrocholesterol.

Lamb and ram spleen. Ubiquinone was found (approx. 1 mg./100 g. of tissue) and substance SF, but not α-tocopherol.

Pig spleen. Two batches were studied, from young and old pigs respectively, with results similar to those for other species (Table 3). Chromatography carried out on larger batches resulted in the separation of a congener (m.p. 43°) which follows ubiquinone in the elution and precedes cholesterol.

Human pancreas and spleen

Spleen. The spleens were hydrolysed with ethanolic potassium hydroxide solution in the presence of pyrogallol and prepared in the manner already described. Alumina from the top of the column was boiled with ethanol to yield some

Table 3. *Comparison of spleens of various species: constituents of unsaponifiable matter*

Animal	*Unsaponifiable material (% wet wt.)	Ubiquinone (mg./100 g.)	Sterol (g./100 g.)	7-Dehydrocholesterol (mg./100 g.)	Formylindole (μg./g.)
Calf	0·445	0·3	0·34	0·06	0·37
Ox	0·44	0·9	0·28	0·02	0·8
Sheep	0·25	0·8	0·27	0·02	0·63
Lamb	0·38	1·1	0·26	0·025	0·47
Ram	0·39	1·0	0·22	0·06	0·45
Pig (old)	0·44	1·9	0·29	0·05	0·5
Pig (young)	0·36	1·2	0·24	0·05	0·5

* α-Tocopherol was not found under these conditions.

Table 4. *Comparison of human spleens: constituents of unsaponifiable matter*

Pathological condition	Sterol* (g./100 g. of tissue)	Ubiquinone (mg./100 g.)	α-Tocopherol† (mg./100 g.)	Formylindole (μg./g.)
1. Glioblastoma	0·37	2·9	0·24	0·16
2. Carcinoma	0·24	5·9	0·91	1·5
3. Rheumatoid arthritis	0·25	4·3	Not found	1·7

* 7-Dehydrocholesterol was not found in any of these specimens. Cholesta-3:5-dien-7-one was not found. Prelog, Ruzicka & Stein (1943) obtained it from pig spleen.

† Quaife & Dju (1949) found 1·3 mg./100 g. of tissue in the spleen of a normal male aged 30.

substance SF and xanthophylls. The fraction containing ubiquinone and α-tocopherol was left in contact with cold methanol for some hours. The methanol dissolved the α-tocopherol and a clean separation from ubiquinone was effected. The results are shown in Table 4.

Pancreas. Two specimens of pancreas were studied in detail (Table 5). Ubiquinone was present in approximately normal amount but α-tocopherol and 7-dehydrocholesterol were not detected. This exploratory work on human tissue is no more than a beginning and although a positive finding is perhaps significant a negative result may not be so.

Nature of substance SF

Concentrates of substance SF dissolved in *cyclo*hexane show absorption peaks at 235 and 280–282 mμ with an inflexion at 250–255 mμ. In ethanol the peaks are displaced to 242 and 292 mμ and the inflexion to 260 mμ. The absorption is changed by alkali, and the fact that in spite of this substance SF is extracted from the alkaline hydrolysate of tissue calls for study.

Substance SF is in fact characteristic of the lipid fraction. Thus trimmed sheep pancreas was comminuted in an electric mixer in the presence of acetone. The mixture was centrifuged and the supernatant liquor was poured off. The residue was refluxed with fresh acetone, centrifuged and the process was then repeated. The combined acetone extracts were diluted with water and extracted with ether and the ether extracts were washed and dried and the solvent was removed. This lipid was saponified in the usual way. The protein residue was twice refluxed with ether and centrifuged. The combined ether extracts were washed and the lipid was saponified separately. The protein residue was then refluxed with ethanol, the mixture was centrifuged, the supernatant liquid was removed, reduced to small bulk and saponified. Finally, the protein residue was itself hydrolysed with alkali and the unsaponifiable matter was extracted. Pyrogallol was added in all four saponifications. Each unsaponifiable fraction was partitioned between light petroleum and 80% (v/v) ethanol, 0·02 M with respect to sodium ethoxide (the correct weight of sodium metal was dissolved in ethanol and the water added afterwards). The ethanolic layer was examined for selective absorption in the regions around 265 and 325 mμ, a peak at 326 mμ indicating the presence of 3-formylindole (except in liver tissue, where vitamin A can interfere).

In this experiment 3-formylindole was found only in the unsaponifiable matter derived from the first (acetone) lipid extract. It does not appear from this to be an artifact of tissue protein.

A simple method of testing tissue for 3-formylindole is thus to extract the comminuted material thoroughly with acetone (at least three extractions) and to examine the unsaponifiable fraction of the extracted lipid in the manner described. By this method it may be shown that kidney tissue contains very little formylindole and heart tissue contains none.

If the unsaponifiable matter is dissolved in light petroleum (b.p. 40–60°) and chromatographed on sucrose (icing sugar plus 3% of starch) most of the material is eluted by light petroleum, whereas substance SF is eluted by 2–4% of acetone in light petroleum.

Provisional unit. The working criterion for substance SF is $\lambda_{max.}$ 282 mμ in *cyclo*hexane and the unit used is $E^{1\%}_{1\,cm.}$ (282 mμ/mg.); thus a fraction showing $E^{1\%}_{1\,cm.}$ (282 mμ) 15 and weighing 12 mg. would contain 180 units. The amounts found have, however, been expressed in the tables in terms of 3-formylindole.

Partition experiments. It was found to be worth while to dissolve crude SF fractions in a mixture of light petroleum (10 vol.) and absolute ethanol (8 vol.) and then to add water (2 vol.). After shaking for 5 min. the layers were separated and substance SF was markedly concentrated in the ethanol layer. When an SF concentrate ($\lambda_{max.}$ 294 mμ in ethanol, $E^{1\%}_{1\,cm.}$ approx. 85) was distributed between light petroleum and 80% ethanol, made 0·02 N with respect to alkali, the light petroleum removed unwanted material and when the ethanol layer was acidified and extracted with ether a material rich in substance SF was obtained, part of it soluble in *cyclo*hexane ($\lambda_{max.}$ 282 mμ; $E^{1\%}_{1\,cm.}$ 96) and part insoluble ($\lambda_{max.}$ 295, 242 mμ; $E^{1\%}_{1\,cm.}$ 231 and 268 respectively).

Micro-high-vacuum distillation of SF fractions obtained by chromatography. The apparatus described by Linstead, Elvidge & Whalley (1955) was used and the vacuum was obtained by means of an oil pump backing a mercury-vapour pump giving 4×10^{-5} mm. Hg. The material used

Table 5. *Comparison of human pancreas: unsaponifiable matter*

Pathological condition	Unsaponifiable material (g./100 g.)	Sterol (g./100 g.)	Ubiquinone (mg./100 g.)	Formylindole (μg./g.)
1. Haemorrhage (Female, 62 years)	0·65	0·23	2·3	Detected
2. Carcinoma (Female, 23 years)	0·76	0·22	2·6	1·75
3. Syphilitic aortitis (Male, 56 years)	0·47	—	—	—

(α-Tocopherol and 7-dehydrocholesterol absent.)

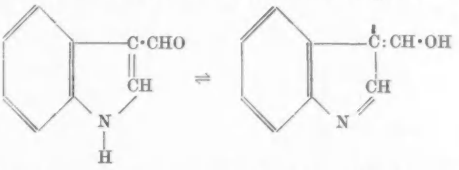
[$E^{1\%}_{1\,cm.}$ (280 mμ) 45·7, 120 mg.] was from pig spleen. The apparatus was heated by means of a glycerol bath and various fractions were collected. At 125–130° pale-yellow crystals were formed on the cold-finger surface ($\lambda_{max.}$ 242, 294, 260 mμ; $E^{1\%}_{1\,cm.}$ 354, 260, 262 respectively). At 145° (60 min.) 6·6 mg. of pale-yellow oil was obtained; it showed the same absorption bands at about one-quarter of the above intensity. Later fractions showed only feeble selective absorption and the residue, a dark-red oil, was of little spectroscopic interest. The first fraction was dissolved in ethanol and taken to dryness in a centrifuge tube. *cyclo*Hexane dissolved part of the material and left some solid behind. The *cyclo*hexane-soluble material was a yellow oil [$\lambda_{max.}$ (ethanol) 240 and 294 mμ; $E^{1\%}_{1\,cm.}$ 207 and 111]. Redistillation (70°, 4×10^{-5} mm Hg) effected little or no improvement and the product seemed somewhat unstable. The *cyclo*hexane-insoluble fraction showed in ethanol $\lambda_{max.}$ 242, 260, 295 mμ, $E^{1\%}_{1\,cm.}$ 755, 650, 773; in ethanolic sodium ethoxide $\lambda_{max.}$ 266, 326 mμ, $E^{1\%}_{1\,cm.}$ 1156 and 1095. The fractions distilling at 175–190° showed $\lambda_{max.}$ 282 mμ, $E^{1\%}_{1\,cm.}$ 40–60 in ethanolic sodium ethoxide; they may well have contained decomposition products.

Other chromatographic SF fractions were similarly distilled. Finally five distillates (all $E^{1\%}_{1\,cm.} > 100$) were combined and redistilled. Two fractions were collected at 70°, $E^{1\%}_{1\,cm.}$ (294 mμ) 222 and 596.

Mass spectra. The mass spectra of the two fractions were kindly obtained for us by Mr A. Quayle of The Thornton Research Laboratory. The fraction with less substance SF showed 'parent' peaks at 392 and 394 with homologues at 406 and 408, although no very strong peak was shown beyond m/e (mass/charge) 177. The purer fraction showed fewer peaks but intense ones occurred at 88, 89, 116, 117, 130, 131 and 144, 145. Small peaks occurred at 256 and 284. The sample contained nitrogen and Mr Quayle considered that the pairs of peaks of similar height were characteristic of nitrogen-containing compounds such as indoles.

Chemical tests. These were carried out on the best distillates. (i) The compound was not oxidized by silver oxide; (ii) it was readily acetylated (acetic anhydride and pyridine) to give a new spectrum; (iii) it gave a silver mirror with ammoniacal silver nitrate. These clues led to indole-aldehyde, and a search of the literature revealed that 3-formylindole very closely resembled the crystalline SF distillate.

The spectra of authentic 3-formylindole in water and in aqueous sodium hydroxide solution were described by Smith (1954) and by Ray & Thimann (1956; in an unstated solvent, probably water). Ray & Curry (1958) described its absorption spectrum in ethanol (see Table 6). Its spectrum in concentrated sulphuric acid is shown in Fig. 1. Fawcett, Taylor, Wain & Wightman (1958) noted that, in the oxidation by plant tissues of indol-3-yl-acetonitrile, 3-formylindole was formed. They pointed out that it could behave as 3-hydroxy-methyleneindolenine, derivatives of both forms being known:



We have examined authentic 3-formylindole and this isomerism fits the effect of alkali.

Fig. 2 shows good isosbestic points characteristic of a two-component system with the equilibrium almost entirely to one side in ethanol and almost completely to the other side in 0·1 M-alkali.

The extraction of the aldehyde from alkaline solutions by means of ether is a slow process and if the solution is almost neutralized a further yield of 3-formylindole can be obtained.

Other substances isolated. (i) In the course of concentrating substance SF from pig pancreas two other materials were isolated. (*a*) White crystals

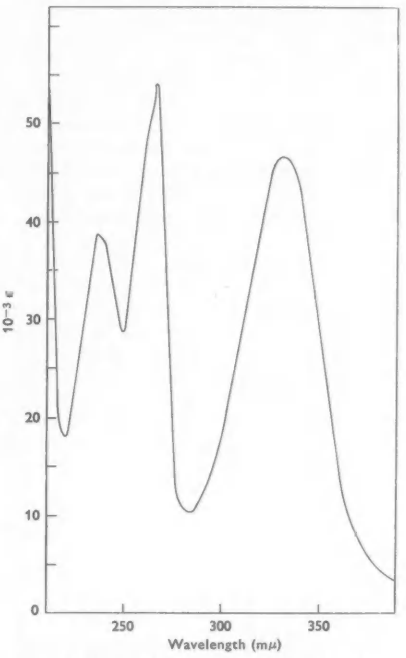


Fig. 1. Absorption curve for 3-formylindole in concentrated sulphuric acid.

(from ethanol), m.p. 131°, no selective absorption of ultraviolet light. Absorption spectrum in conc. sulphuric acid $\lambda_{max.}$ 318 and 416 mμ, $E^{1\%}_{1\,cm.}$ 96 and 33 respectively. [Found: C, 78·82, 78·75; H, 11·40, 11·49; O, by diff., 9·77 % mol. wt. (Rast) 306, 327. $C_{22}H_{38}O_2$ requires C, 78·98; H, 11·45; O,

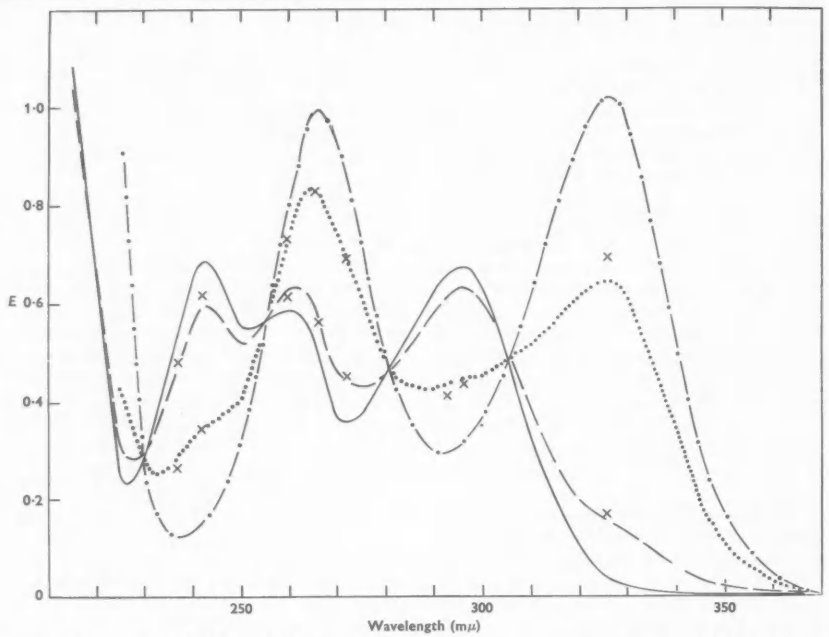


Fig. 2. Absorption curves for 3-formylindole. ——, In ethanol; –·–·, in aqueous ethanol, 0·1 M with respect to NaOH; ....., in aqueous ethanol, 5 mM with respect to NaOH; – – –, in aqueous ethanol, mM with respect to NaOH. ×, Represents a calculated point, assuming a two-component system.

Table 6. *Absorption characteristics of 3-formylindole and its N-acetate*

Solvent	$\lambda_{max.}$ (mμ)	$\epsilon_{max.}$	$\lambda_{min.}$ (mμ)	$\epsilon_{min.}$
	3-Formylindole			
Water	210	27 500	230	6 300
	245	12 600	255	10 700
	262	11 750	277	6 750
	302	13 800	—	—
Ethanol	242	13 700	227	4 700
	260	11 700	252	11 000
	296	13 450	272	7 200
0·02 N-NaOH	~ 250	6 300	235	3 560
	266	17 700	290	6 000
	327	16 000	—	—
Ethanolic sodium ethoxide	266	19 900	237	2 500
	326	20 400	293	5 840
Conc. H_2SO_4	235	38 800	220	17 900
	265	54 200	250	28 700
	329	46 700	285	10 100
	3-Formylindole *N*-acetate			
Ethanol	218	31 300	268	6 300
	245	13 600	—	—
	260	6 500	—	—
	298	13 440	—	—

9·57 %; mol.wt. 334]. Liebermann–Burchardt and Zimmermann tests were positive. Infrared absorption in CS_2 3610 (sharp, weak), 2924, 2865, 1745 (sharp, strong), 1377 (~1370), 1166 (~1155), 1050, 1020, 954, 798 cm.$^{-1}$. Infrared data suggest that the compound is a keto steroid with a hydroxyl group, a carbonyl group in the five-membered ring, but this hypothesis does not fit the analysis. Further work is needed. (*b*) Pale-yellow crystals were obtained from crude preparations of substance SF from pig pancreas. On recrystallization traces of SF were removed. After five crystallizations the m.p. was 82° (sharp). Unsaturation was indicated by immediate decolorization of a solution of bromine in carbon tetrachloride. The substance readily absorbed oxygen as will be seen from two analyses [Found: C, 77·25, 76·98; H, 12·9, 12·3; O, by diff., 9·84, 10·72; mol.wt. 511 Rast. A later analysis gave C, 75·58; H, 12·28; O, by diff., 12·14 %, and later still C, 72·21; H, 12·23; O, by diff., 15·56; mol.wt. (Rast) 516. $C_{33}H_{64}O_3$ requires C, 77·88; H, 12·68; O, 9·46 %, $C_{33}H_{64}O_4$ requires C, 75·5; H, 12·30; O, 12·20 %, $C_{33}H_{64}O_5$ requires C, 73·33; H, 11·95; O, 14·80 %]. After further recrystallization in the presence of animal charcoal the m.p. rose to 86·5°. The infrared absorption showed a strong sharp band due to hydroxyl at 3332 cm.$^{-1}$ in liquid paraffin and 3460 cm.$^{-1}$ in chloroform. Other strong bands occurred at 2915, 2857, 1642, 1613, 1563, 1543, 1462, 1372 and 716 cm.$^{-1}$. The compound probably contains ethylenic linkages, and it may be an unsaturated glycerol ether, $C_{30}H_{57}\cdot O\cdot CH_2\cdot CH(OH)\cdot CH_2\cdot OH$.

(ii) A third compound from pig pancreas was obtained from ubiquinone fractions. From its infrared spectrum it was an aliphatic ketone or aldehyde (1736, 2915, 2849, 1468, 1464, 1193, 1171, 1148, 1107, 1094, 718 cm.$^{-1}$). The compound showed weak selective absorption at 270 mμ; m.p. 43°.

Further work is needed on all three substances. They are only incidental to the present study.

DISCUSSION

Ubiquinone at the concentration of 1–2 mg./100 g. of tissue occurred in the pancreas of ox, sheep, pig and man. It was also found in lymph glands embedded in the pancreatic tissue and it was also present in spleen. α-Tocopherol, found in sheep pancreas, was either absent from pancreas of ox and pig or present in much lower concentration. Tocopherol was not detected in animal spleen but was found in human spleen. Cholesterol obtained from spleen contained detectable amounts of 7-dehydrocholesterol (0·02–0·06 mg./100 g.), but this substance was lacking in the sterol from pancreas.

The amount of ubiquinone found in human spleen may possibly exceed that found in animals, but more tests are needed. Similarly, the absence of 7-dehydrocholesterol needs to be confirmed on more specimens.

The detection of 3-formylindole is interesting. Salhanick & Berliner (1957) studied a large feminizing adrenal carcinoma from a man aged 22, from which they isolated progesterone, equilenin and five unidentified materials, two of which were designated AAE-I-AI and AAE-I-BI. Salhanick & Berliner (1957) recorded for AAE-I-AI absorption peaks at 242, 260 and 295 mμ in methanol and at 240, 265 and 330 mμ in concentrated sulphuric acid. Their published curves display maxima and minima with clear patterns of relative intensities of absorption. The total amount of material available (132 μg.) was very small and there was no evidence about purity. The preparation gave a positive Millon's test (thought to indicate a 3:5-unsubstituted hydroxyphenyl group) and a positive Zimmermann test for 17-oxo steroid. Both tests are now seen to be due to congeners and not to the selectively absorbing entity. There can be no doubt that the latter was indistinguishable from 3-formylindole. This compound shows three maxima and minima in each of two very different solvents and also reproduces the patterns of intensity seen in the diagrams of Salhanick & Berliner (1957).

Ray & Curry (1958) studied the photochemical destruction of indolylacetic acid by monochromatic light (280 mμ) and found 3-formylindole among the products. They quoted Stutz as having observed that enzymic reduction, which does not normally lead to 3-formylindole, does so when cytochrome *c* and a cytochrome oxidase are present in addition to indolylacetic acid oxidase.

As already noted, Fawcett *et al.* (1958) found that plant tissues could form 3-formylindole from indol-3-yl-acetonitrile. Jones & Taylor (1957) detected in cabbage the acetonitrile, indole-3-carboxylic acid as well as 3-formylindole.

The question whether 3-formylindole is as such biologically significant in animal tissues cannot yet be answered. That it is an artifact is very possible. It is not, however, found in all tissues, which may indicate that it is not a normal artifact from the tryptophan of tissue protein. The small yield suggests that if 3-formylindole is an artifact it must arise from a highly specific precursor. This seems to be a good working hypothesis.

SUMMARY

1. Pancreas and spleen from ox, sheep and pig have been found to contain small amounts of ubiquinone. This substance has also been found in human spleen and pancreas. In the course of the work the distribution of α-tocopherol and 7-dehydrocholesterol have been studied.

2. Unsaponifiable fractions from spleen and pancreas contain very small amounts of a substance spectroscopically identical with 3-formylindole.

3. The relationship of the absorption spectra in two solvents of a fraction isolated by Salhanick & Berliner (1957) from a suprarenal tumour to those of 3-formylindole is discussed.

We wish to express our gratitude to the Nuffield Foundation for a grant towards the cost of investigation. We are indebted to the Thornton Research Laboratory for mass spectra, to Professor H. L. Sheehan for post-mortem material and to Dr J. B. Jepson for 3-formylindole.

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Unsaponifiable Fraction of Lipid from Normal and Diseased Human Kidney

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Lowe, Morton & Vernon (1957) reported on the unsaponifiable constituents of kidneys from a number of species. Vitamin A is normally present, but in amounts so small that the fractionation of kidney unsaponifiable matter is not hindered by the presence of artifacts derived from vitamin A. The two substances SA and SC (Heaton, Lowe & Morton, 1955, 1957) were present in kidneys, the amounts being of the order 20 μg./g. for SA and 5 μg./g. for SC, on the assumption that the best preparations so far obtained are not far from pure. Vitamin E was detected in the kidneys of dog, sheep and cat. It was found that the vitamin A content of cat kidney was very much greater than that of kidney from other species; similarly, the vitamin E content was quite unusually high. In the cat kidney vitamins A and E were found predominantly in the cortex and vitamin E was not detected in the medulla.

Much of the material used by Lowe *et al.* (1957) was from healthy animals which had been killed only a very short time before the kidney tissue was saponified. Post-mortem changes were therefore usually small and often minimal.

The study of human kidney tissue offers both advantages and disadvantages. On the debit side is the fact that the kidney tissue which becomes available through the co-operation of pathologists has often had time to undergo change, and moreover usually comes from a person who had died of disease rather than accident. Among the advantages of studying human tissue is the fact that very full medical records may often be consulted so that any abnormalities in the chemical constituents of kidney tissues can be considered in the light of clinical and pathological observations.

The present investigation is largely exploratory. The structures of substances SA and SC, as well as their biological significance, are the subjects of parallel research. The name ubiquinone has been given to substance SA because it has been shown to be very widely distributed and to occur at sites which imply that it exhibits catalytic properties.

It has a high oxidation–reduction potential and behaves like a typical quinone (Morton, Wilson, Lowe & Leat, 1957).

MATERIALS AND METHODS

Human kidneys were obtained through the help of Professor H. L. Sheehan, Dr A. H. Cruickshank and their colleagues in the Department of Pathology, University of Liverpool. In addition, kidneys from patients who had died from nephritis were obtained from Dr J. C. Brundret of Sefton General Hospital, Liverpool, Dr F. B. Mooney of St Helens' Hospital, Dr R. M. Rawcliffe of St Catherine's Hospital, Birkenhead, Dr R. E. Newell of the Royal Liverpool Children's Hospital and Dr M. J. J. Wodzinski of Walton Hospital, Liverpool. Human blood was obtained through the aid of Dr D. Lehane of the Regional Blood Transfusion Service.

As soon as kidneys were received, the perinephric fat and membrane were removed, together with most of the pyramid. Each kidney was cut longitudinally and the halves were washed in water, dried roughly on filter paper and weighed. If it was not possible to proceed at once the tissue was covered with ethanol and left at $-20°$.

Saponification. The tissue was cut into small pieces and digested on the water bath with 60% (w/v) KOH (0·5 ml./g. of tissue) until the mixture became homogeneous; ethanol (1 ml./g. of tissue) was then added and the mixture boiled under reflux for 30–45 min. It was later found that it is better to mix the 60% KOH and ethanol (with dissolved pyrogallol) before bringing the tissue into contact with alkali; this minimizes loss of vitamin E. After cooling, water (2 vol.) was added and the solution was then extracted six times with ether (dried over sodium and then freshly distilled over reduced iron). The combined ether extracts were washed free from alkali, dried over anhydrous Na_2SO_4, filtered through a sintered-glass funnel, evaporated to dryness under nitrogen and weighed.

Determination of vitamin A. This was carried out on small portions of the unsaponifiable matter (30–40 mg.) with the antimony trichloride reaction (Glover, Goodwin & Morton, 1947).

Chromatography. The unsaponifiable matter was chromatographed on alumina (Brockmann grade 3), as described in previous papers (Heaton *et al.* 1957; Lowe *et al.* 1957). Grade 0 alumina (P. Spence and Co., Widnes) was partially deactivated with water to Brockmann grade 3, and as a rule 10 g. was taken for each 100 mg. of unsaponifiable matter. The procedure was to dissolve the material in light petroleum (b.p. 40–60°) and to develop the chromatogram with the same solvent. At this stage a small amount of hydrocarbon (inflexions at 235 and 260 mμ) was eluted. The chromatogram was further developed with 2% (v/v) of ether in light petroleum, which eluted any remaining aliphatic hydrocarbons, lycopene, carotene and probably any cholesta-3:5-dien-7-one. Ubiquinone was eluted by means of 4% (v/v) of ether in light petroleum and tocopherol by 6% (v/v) of ether in light petroleum, but the separation was not always sharp. Any remaining tocopherol was eluted by means of 8% (v/v) of ether in light petroleum. Cholesterol, together with substance SC, 7-dehydrocholesterol and vitamin A were eluted by 20% (v/v) of ether in light petroleum. It was important to persist until the eluate (after removal of solvent) ceased to give a positive Liebermann–Burchard test. Ether (100%) eluted small quantities of material containing any xanthophylls and α-tocopherylquinone.

The sterol fraction was then acetylated (pyridine and acetic anhydride) and the mixture of acetates chromatographed. Most of the cholesteryl acetate was eluted by 1% (v/v) of ether in light petroleum. This fraction also contained 7-dehydrocholesteryl acetate and vitamin A acetate. As soon as the eluate ceased to give a positive Liebermann–Burchard test the eluting solvent was changed to 4% (v/v) of ether in light petroleum and SC acetate came through the column. Elution of this material was completed by means of 8% of ether in light petroleum; 100% ether eluted SC alcohol.

Determination of tocopherol. Tocopherols are sensitive to alkali (Moss, Cuthbertson, Danielli & Drummond, 1938) but saponification of oils in the presence of pyrogallol (Tosic & Moore, 1943) diminishes the loss. Quaife & Dju (1949) determined tocopherol in tissue by first extracting the lipid and then concentrating the vitamin E by molecular distillation. It was important in the present work to know how much tocopherol was destroyed in the alkaline digestion of kidney tissue, how much survived the chromatography, and which were the principal decomposition products.

The method of Emmerie & Engel (1938) was applied to chromatographic fractions which showed signs of selective absorption in the region 290–300 mμ.

Portions of 0·5, 0·75, 1·0, 1·5, 2·0 and 2·5 ml. of a standard solution of α-tocopherol (Roche Products Ltd.) in ethanol (0·24 mg./ml.) were measured into separate 25 ml. volumetric flasks. To each was added 1 ml. of $FeCl_3$ solution (0·2%, w/v, in absolute ethanol) and 1 ml. of αα-dipyridyl solution (0·5%, w/v, in absolute ethanol, freshly prepared). The volume was made up to 25 ml. with ethanol and after 30 min. the intensity of absorption of each solution was measured at 520 mμ with a compensating cell containing a similar solution but with omission of the tocopherol. The calibration curve was a straight line passing through the origin, and if x = mg. of tocopherol and $E = \log I_0/I$ for a 1 cm. cell at 520 mμ, $x = 0{\cdot}8E$.

RESULTS

Trial experiments

Experiment 1. A large 'normal' kidney was cut longitudinally and laterally to give four nearly equal parts. One portion was saponified in the usual way and the unsaponifiable matter was chromatographed. A second portion was saponified in the presence of 0·4 g. of pyrogallol and the unsaponifiable matter chromatographed. Tocopherol was added in known amounts to the third and fourth portions, which were then treated in the same way as the first two.

Table 1 (*a*) shows that the recovery of added tocopherol is far from satisfactory. It is interesting to note that the yield of ubiquinone is about doubled by the use of pyrogallol but that of substance SC is unaffected.

Experiment 2. A 'normal' kidney was divided longitudinally into two equal parts and to each was

added the appropriate volume of 60% KOH, pyrogallol solution and 4·5 mg. of tocopherol. Both were digested on the water bath until the tissue had all dissolved (8 min.). One portion was extracted at once and the other was refluxed with absolute ethanol before extraction. In both cases the tocopherol found was about 57% of that added. There was no difference in the ubiquinone content observed. From this experiment it was clear that the loss of tocopherol occurred at an early stage and was not made worse by refluxing with ethanol.

Experiment 3. A large 'normal' kidney was divided longitudinally and laterally to give four nearly equal parts. In this test the point at issue was whether contact with concentrated aqueous alkali is the destructive agency. Table 1 (*b*) shows beyond doubt that the 60% KOH and the ethanol (containing pyrogallol) must be mixed first and that undiluted 60% KOH is unsuitable. The recovery of added tocopherol can now be raised to 88%. This procedure seems also to permit optimum extraction of ubiquinone and substance SC.

Experiment 4. It was found that tocopherol was absent from the human kidney cortex, but the technique used probably involved considerable loss of tocopherol. Accordingly, the findings of expt. 3 were applied to a further test of this point. A 'normal' kidney was divided longitudinally into two parts, one of which was dissected to give medulla and cortex. The other half was 'saponified' whole. The alkali, premixed with ethanol containing pyrogallol, was added to the tissue in each case. The unsaponifiable fractions were chromatographed; elution with 4% (v/v) of ether in light petroleum removed ubiquinone and tocopherol was eluted with 6% and 8% (v/v) of ether in light petroleum. Tocopherol was found in the fractions from medulla and from the half-kidney but not in the cortical extracts ($\lambda_{max.}$ 292 and 300 mμ). The

Table 1

(*a*) *Effect of pyrogallol as antioxidant on the analysis of kidney tissue with and without added tocopherol*

Four portions of one 'normal' kidney were used. Arbitrary units: $E^{1\%}_{1\,cm.}$ 272 mμ (ubiquinone) or 275 mμ (substance SC) multiplied by weight of chromatographic fraction in mg. If $E^{1\%}_{1\,cm.}$ (272 mμ) is 170 for ubiquinone, 1 unit = 6 μg. of ubiquinone; if $E^{1\%}_{1\,cm.}$ (275 mμ) is 90 for 'pure' substance SC, 1 unit = 11·1 μg. of substance SC.

Wt. of tissue (g.)	Pyrogallol added (g.)	Tocopherol added (mg.)	Tocopherol found (mg.)	Ubiquinone found (units/g. of tissue)	Substance SC found (units/g. of tissue)
42	Nil	Nil	0·22	2·16	1·0
41	0·4	Nil	0·30	4·01	0·96
40	Nil	3·8	1·72	2·18	0·97
40	0·4	4·0	2·24	4·32	0·95

(*b*) *Effect of saponification procedure, with four portions of the same kidney*

(i) Tissue digested with 60% KOH containing pyrogallol, followed by refluxing with ethanol for 30 min. (ii) Tissue digested under reflux for 30 min. with premixed 60% KOH and ethanol and pyrogallol. (iii) α-Tocopherol added; digestion was as described in (i). (iv) α-Tocopherol added, digestion was as described in (ii).

Portion	Wt. of tissue (g.)	Pyrogallol added (g.)	Tocopherol added (mg.)	Tocopherol found (mg.)	Recovery of added tocopherol (%)
(i)	38	0·4	Nil	0·30	—
(ii)	37	0·4	Nil	0·87	—
(iii)	37	0·4	4·8	2·74	50·8
(iv)	39	0·4	4·8	5·09	88

Table 2. *Concentrations of tocopherol in human kidney cortex and medulla and whole kidney*

One kidney was divided longitudinally; one half was divided into cortex and medulla.

	Cortex	Medulla	Whole tissue
Wt. of tissue (g.)	37	31	70
Tocopherol (mg.)			
in 6% ether–light petroleum fraction	Absent	0·20	0·18
in 8% ether–light petroleum fraction	Absent	0·60	0·65
		0·80	0·83
Concn. (mg./100 g.)	—	2·54	1·18

amounts of tocopherol were determined by the Emmerie–Engel method (Table 2). The results confirm the absence of tocopherol from the cortex of the 'normal' human kidney.

'Normal' subjects

The first stage in the present study was to examine human kidney tissue from ten subjects. This was followed by the examination of kidney tissue from four other subjects so as to reveal any differences between cortex and medulla. Table 3 gives the age, sex and cause of death for the subjects, with the analytical findings on the kidneys. Table 4 gives the results for whole kidney cortex and medulla from four subjects. [The third kidney studied gave intractable emulsions during the extraction process; the results obtained are included in the table but were not used in obtaining average values.]

It will be seen that the percentage of unsaponifiable matter (average 0·33) and the vitamin A content (average 3·5 i.u./g.) vary over quite narrow ranges. Ubiquinone is present in every case (average 3·65 units/g. or perhaps 1·5–3·0 mg./kidney) and so is substance SC (average 1·2 units/g.). Again the ranges are narrow, but the result from a boy aged 7 years killed in an accident is exceptional; here substance SC was just detectable. Tocopherol was also found in every case, but from the results it seems certain that the average figure of 0·48 mg./100 g. of kidney is too low and that if the improved method of digestion of tissue had been used throughout, 1 mg./100 g. of kidney tissue would be a better estimate. Mason & Dju (1953) obtained results agreeing better with the revised estimate of about 1 mg./100 g. The sterol content (average 0·23 g./100 g. of tissue) varies, but not very widely.

Abnormal kidneys

Sixteen specimens of kidney tissue from subjects with kidney disease have been studied. Table 5 records the results, the specimens being listed in the order that they were received.

In every case the kidney tissue was treated according to the revised procedure whereby destruction of tocopherol was minimized. The kidney was cut into small pieces, and an equal weight of absolute ethanol was added. Pyrogallol solution (0·5%, w/v, in ethanol) was then added (1 ml./5 g. of tissue). Then 60% (w/v) KOH was added (0·5 ml./g. of tissue). The whole was refluxed on the water bath for 45 min., after which it was cooled, water added and extraction with ether carried out in the ordinary way.

The condition shown in the table for each specimen was assigned to it by the pathologist concerned, in the light of the clinical history and (usually) of the histological findings. Three types were studied, namely chronic, acute and sub-acute chronic (large white kidney) nephritis. Chronic nephritis

Table 3. *'Normal' human kidneys*

No.	Sex and age (years) of subject		Cause of death	Wt. of kidney (g.)	Percentage of unsaponifiable matter	Vitamin A (i.u./g.)	Ubiquinone (units/g. of kidney)*	Substance SC (units/g. of kidney)*	Tocopherol (mg./100 g. of kidney)	Sterol (g./100 g. of kidney)
1	M.	57	Coronary thrombosis	140	0·272	2·8	3·24	1·71	0·72	0·165
2	F.	44	Cerebral haemorrhage	108	0·301	4·7	2·00	1·20	0·80	0·127
3	M.	59	Coronary thrombosis	133	0·125†	1·3	0·80	0·95	0·11	0·093
4	M.	59	Peritonitis	112	0·32	1·7	4·95	1·43	0·34	0·173
5	F.	75	Accident	138 (two)	0·309	2·2	3·67	0·62	0·36	0·251
6	M.	51	Carcinoma of stomach	89	0·29	2·1	4·07	1·96	0·41	0·195
7	F.	56	Cardiac failure (arterio-sclerotic granularity shown by kidney)	102	0·30	6·8	2·07	1·24	0·40	0·220
8	F.	72	Internal haemorrhage	75	0·39	3·9	3·21	0·91	0·61	0·261
9	F.	20	Heart failure	150	0·24	4·0	1·70	0·27	0·56	0·181
10	F.	63	Heart failure	116	0·39	3·9	4·70	1·80	0·73	0·284
11	M.	78	Heart failure	77	0·395	3·6	5·10	1·18	0·60	0·271
				85	0·375	3·0	4·00	0·68	0·50	0·245
12	M.	63	Heart failure	127	0·34	6·2	4·50	2·37	0·37	0·257
13	M.	52	Coronary thrombosis	55·5	0·34	1·7	3·90	0·85	0·37	0·241
14	M.	7	Accident	40	0·396	2·7	4·00	Trace only	0·29	0·272
			Average (excluding no. 3)		0·33	3·5	3·65	1·2	0·48	0·23

* See Table 1 for explanation of units.

† Low yield was due to intractable emulsions.

Table 4. *Comparison of the unsaponifiable constituents from whole human kidney and from kidney cortex and medulla*

One kidney from each of subjects 11, 12, 13 and 14 was analysed whole, the other after separation into cortex and medulla; from the latter results, the values for the whole second kidney were calculated (column 3). The results for each set of four kidneys have been averaged.

	Whole kidney	Calc. for other kidney	Cortex	Medulla
Wt. of tissue (g.)	300·5	301·5	152·5	149
Percentage of unsaponifiable matter	0·368	0·369	0·395	0·344
Vitamin A concentration (i.u./g.)	3·5	2·5	3·4	1·8
Percentage of unsaponifiable matter recovered after chromatography	84	79	80	77
Ubiquinone (units/g.)	3·8	4·7	5·6	3·8
Substance SC (units/g.)*	1·5	1·2	1·7	0·8
Tocopherol (mg./100 g. of tissue)	0·42	0·37	Absent	0·74
Sterol (g./100 g. of tissue)	0·26	0·26	0·27	0·26

* Excluding subject no. 14 (Table 3), where substance SC was present in negligible amount.

is the most common of the three conditions, but we were fortunate in obtaining good specimens of kidneys characteristic of the other two states. Three cases may be described as examples.

Subject 15 was a man aged 46, who died of subacute chronic nephritis. The kidney was big and the cortex was very light in colour and enlarged. The medulla was dissected from the cortex and the two tissues were examined separately.

When cortical unsaponifiable matter (209 mg.) was chromatographed, light petroleum eluted 5·4 mg. of material showing an inflexion near 260 mμ and 2 % (v/v) of ether in light petroleum eluted 1·8 mg. of material containing carotene. When 4 % (v/v) of ether in light petroleum was used, a small amount (0·5 mg.) of material with $\lambda_{max.}$ 270 mμ was eluted and the absorbing entity was found to be cholesta-3:5-dien-7-one (Kantiengar & Morton, 1955). When the eluting solvent was 6 % (v/v) of ether in light petroleum ubiquinone was removed. The vitamin A and cholesterol (177 mg.) were eluted by means of 20 % (v/v) of ether in light petroleum. Ether eluted a further 13 mg. of material. The sterol fraction was esterified with pyridine and acetic anhydride and the acetate was rechromatographed. The fractions contained 7-dehydrocholesterol and vitamin A in small traces but substance SC was not found.

Medullary unsaponifiable matter (202 mg.) was similarly studied. Ubiquinone was found but no cholesta-3:5-dien-7-one. The fraction (3·6 mg.) eluted by means of 6 % (v/v) of ether in light petroleum showed $\lambda_{max.}$ 290 mμ with a secondary peak at 298 mμ ($E^{1\%}_{1\,cm.}$ 7·08, 6·87). The absorbing substance was tocopherol. A further, smaller amount of tocopherol was eluted by means of 8 % (v/v) of ether in light petroleum. With 20 % (v/v) of ether in light petroleum cholesterol (152 mg.) was eluted together with traces of 7-dehydrocholesterol and vitamin A. Ether eluted 1 mg. of additional adsorbate. Esterification of the sterol fraction, followed by chromatography, showed substance SC to be absent. The amounts of ubiquinone found were 1·64 and 1·0 unit/g. of medulla and cortex respectively. Substance SC was absent from both types of tissue and tocopherol found to the extent of 0·92 mg./100 g. of medulla was absent from the cortex. A trace of dienone was found in the cortex but not in the medulla.

Subject 16 was a woman aged 79 who died of chronic nephritis. Although the demarcation of cortex and medulla was ill-defined, the two tissues were separated as well as was possible and they were studied separately.

Cortical unsaponifiable matter (92 mg.) was chromatographed in the usual way. The sole absorbing entity eluted by 4 and 6 % (v/v) of ether in light petroleum was ubiquinone. The sterol eluted by 20 % (v/v) of ether in light petroleum was acetylated and rechromatographed. Traces of 7-dehydrocholesterol and vitamin A were found but substance SC was absent. The ubiquinone contents were 3·6 and 4·14 units/g. for the medulla and cortex respectively.

Subject 17 was a woman aged 55; there was some arteriosclerosis of the kidneys but no nephritis. The kidney was cut longitudinally and one half was divided into medulla and cortex. Chromatography of the half-kidney unsaponifiable matter (440 mg.) gave with 4 % (v/v) of ether in light petroleum a good ubiquinone fraction [2·2 mg., $E^{1\%}_{1\,cm.}$ (272 mμ) 85·5] and with 6 % (v/v) of ether in light petroleum the absorbing material contained ubiquinone and tocopherol. The main part of the tocopherol ($\lambda_{max.}$ 290 and 300 mμ) was, however, eluted with 8 % (v/v) of ether in light petroleum. The sterol fraction, eluted by means of 20 % (v/v) of ether in light petroleum, was examined and found to contain a little substance SC and vitamin A; it was then acetylated and rechromatographed. The major part of the material was eluted with 1 % (v/v) of ether in light petroleum and this contained

Table 5. *Abnormal human kidneys*

No.	Sex	Age (years) of subject	Diagnosis	Wt. of kidney (g.)	Percentage of unsaponifiable matter	Vitamin A (i.u./g. of tissue)	Ubiquinone (units/g. of kidney)†	Substance SC (units/g. of kidney)†	Tocopherol (mg./100 g. of kidney)	Sterol (g./100 g. of tissue)	Tocopheryl-quinone (mg./100 g. of kidney)
15	M.	46	Subacute chronic nephritis; large kidney, pale enlarged cortex	195	0·56	0·4	1·3	Absent	0·46	0·383	Absent
16	F.	79	Chronic nephritis	43	0·55	6–7	3·87	Absent	Trace	0·315	Absent
17	F.	55	Some arteriosclerosis of kidney	72	0·43	2·8	8·2	1·35	0·75	0·292	0·75
19	M.	68	Uraemia, chronic nephritis	51	0·35	2·1	2·27	0·45	1·27	0·205	Absent
20	F.	—	Acute nephritis*	143	0·35	1·0	2·52	Absent	Absent	0·244	0·14
21	F.	—	Acute nephritis*	144	0·55	1·2	1·55	Absent	Absent	0·301	0·20
22	M.	66	Chronic nephritis and pneumonia	73	0·29	0·6	2·16	Absent	Trace	0·183	0·41
23	F.	55	Chronic nephritis; kidneys very small	41·5	0·42	2·9	2·70	Absent	Absent	0·249	0·44
24	M.	3	Sub-acute chronic nephritis; large pale kidneys, typical	80 (two)	1·06	1·2	4·97	Absent	0·46	0·548	Absent
25	M.	50	Chronic nephritis*	87	0·43	2·6	3·69	Absent	1·28	0·350	Absent
26	M.	39	Chronic glomerular nephritis*	125 (two)	0·40	1·5	3·82	Absent	1·82	0·269	Absent
27	M.	67	Sub-acute chronic nephritis	165	0·61	2·0	5·4	Absent	0·86	0·508	Absent
28	M.	73	Chronic hypertensive nephritis	109 (two)	0·32	0·9	4·77	Absent	1·92	0·215	Absent
29	M.	80	Mild chronic hypertensive nephritis with granular scarring and some adhesion of the capsules	152 (two)	0·42	2·0	2·82	Absent	0·70	0·242	Absent
30	F.	42	Lipoid nephrosis	160	1·23	2·2	5·53	Absent	Absent	1·042	0·10
31	M.	42	Terminal chronic nephritis	36	0·46	1·6	7·37	Absent	0·72	0·290	0·15

* Confirmed histologically.

† See Table 1 for explanation of units.

cholesteryl acetate plus traces of 7-dehydrosteroid acetate and vitamin A acetate. The eluate with 4% (v/v) of ether in light petroleum showed no selective absorption. The eluate with 8% (v/v) of ether in light petroleum (1·5 mg.) showed the absorption spectrum of SC acetate practically undistorted. Ether eluted a small quantity of tocopherylquinone giving twin peaks at 260 and 270 mμ.

The whole process of obtaining unsaponifiable fractions and chromatographic separation was then carried out both on the cortex and medulla and the results are shown in Table 9.

Determinations of the principal trace constituents were made similarly on other diseased kidneys.

The most striking result (Table 5) is that substance SC is absent from the kidney in all but one of the cases of nephritis. Tocopherol is sometimes absent and sometimes present at concentrations which are not below the normal (say 1 mg./100 g.). When tocopherol is absent, tocopherylquinone can often be detected (five cases).

DISCUSSION

Lowe *et al.* (1957) found that ubiquinone and substance SC were normal constituents of the unsaponifiable matter from kidneys of 11 species, the amounts present being of the order 20 μg./g. for the former substance and 5 μg./g. for the latter. For 13 'normal' human kidneys and 15 nephritic kidneys the average ubiquinone content of 3·65 and 3·64 units/g. respectively are almost identical and correspond with 21·9 μg./g. This agrees very well with the average for other species and it is clear that not only is the range of variation small but ubiquinone also does not vary in kidney disease.

Kidney tissue in most species contains from 1 to 10 i.u. of vitamin A/g. Moore & Sharman (1950) noted that in rats receiving 40 i.u. of vitamin A/day, males but not females consistently stored more vitamin A in the kidneys than in the liver. This is perhaps a special situation, and in fact kidney stores are in most species quite low when liver stores are high. The differences in vitamin A content shown in Tables 4, 5 and 8 are quite small and afford little indication of a direct relationship between kidney disease and kidney vitamin A. The report by Lawrie, Moore & Rajagopal (1941) that nephritic kidneys do not contain vitamin A is not confirmed (Tables 6 and 7).

Lowe *et al.* (1957) found vitamin E in cat, dog and sheep kidney, the concentration in cat kidney being very high compared with the other two species. The total lipid content of cat kidney is high compared with other species (Mottram, 1916; Turner, 1931). Gavins & Morrison (1949) found considerable amounts of lipids in the tubule cells of the nephrons of cats, both by chemical and histological methods. In the cat both vitamins A and E occurred predominantly in the cortex; indeed no vitamin E could be found in the medulla. Vitamin E is present in human kidney to the extent of about 1 mg./100 g., as compared with perhaps 2·5 mg./100 g. for cat kidney. In the human kidney the tocopherol is located in the medulla and none can be found in the cortex (Tables 4 and 9). Tocopherol was detected, in fairly normal amounts, in the kidneys of six out of nine subjects with chronic nephritis. In two cases of acute nephritis no tocopherol could be found in the kidney unsaponifiable matter but quite appreciable amounts of tocopherylquinone were found instead. The pattern of incidence of this quinone in kidney extracts is not easy to explain but it is not, in our view, consistent with the substance being a laboratory artifact of tocopherol.

Table 6. *Comparison of kidney cortex and medulla in a case of sub-acute chronic nephritis*

	Cortex	Medulla
Weight (g.)	130	65
Percentage of unsaponifiable material	0·579	0·519
Vitamin A (i.u./g. of tissue)	0·4	0·4

Table 7. *Comparison of kidney cortex and medulla in a case of chronic nephritis*

	Cortex	Medulla
Weight (g.)	22	21
Percentage of unsaponifiable material	0·465	0·629
Vitamin A (i.u./g.)	7·3	6·0

Table 8. *Comparison of kidney cortex and medulla in a case of arteriosclerosis of kidney*

One kidney was divided into two; one half was divided into cortex and medulla.

	Whole tissue	Cortex	Medulla
Weight (g.)	36	—	—
Percentage of unsaponifiable material	0·434	0·51	0·40
Vitamin A (i.u./g.)	2·8	3·8	1·6

Table 9. *Minor constituents of unsaponifiable matter from cortex and medulla of an arteriosclerotic kidney (subject no.* 17)

	Cortex	Medulla
Ubiquinone (units/g.)*	8·9	7·4
Substance SC (units/g.)*	1·5	1·2
Sterol (g./100 g.)	0·306	0·276
Tocopherol (mg./g.)	Absent	1·5
Tocopherylquinone	Absent	Present

* See Table 1 for explanation of units.

Table 10. *Comparison of the unsaponifiable constituents of 'normal' kidneys and abnormal kidneys of human*

	Percentage of unsaponifiable material	Vitamin A (i.u./g.)	Ubiquinone (units/g.)*	Substance SC (units/g.)*	Tocopherol (mg./100 g.)	Sterol (g./100 g.)
Normal kidneys (thirteen subjects)	0·33	3·5	3·65	1·2	0·48†	0·23
Chronic nephritis (nine subjects)	0·40	2·3	3·72	Absent in eight of nine subjects	Present in six, absent in three out of nine subjects	0·25
Acute nephritis (two subjects)	0·45	1·1	2·0	Absent	Absent	0·27
Sub-acute chronic nephritis (large white kidney; four subjects)	0·86	1·4	4·3	Absent	0·44 (absent in one case)	0·62

* See Table 1 for explanation of units.

† 1·0 is a more likely figure (see text).

In the normal kidney Mason & Dju (1953) found that the vitamin E content was greater for females than males. This is confirmed for the present 'normal' series. In Table 5 it is seen that of the five nephritic kidneys obtained from females four lacked tocopherol altogether and the fifth contained only a trace. On the other hand, all the kidneys from males with nephritis contained (with one exception, subject no. 22) normal amounts of tocopherols. So far as the results go, the disappearance of tocopherol from the kidney is associated with nephritis (any type) in the female; the arteriosclerotic kidney from a woman showed a normal tocopherol content. The evidence points to the possibility that in the female tocopherol disappears in nephritis via the tocopherylquinone.

O'Connor (1955) reported that some cases of acute nephritis responded to tocopherol so that kidney-function tests gave normal results. Massive doses were given, but if excessive breakdown of tocopherol is characteristic of nephritis a large intake might well be necessary in compensatory therapy. Butturini (1955) also claims that tocopherol is beneficial in lipoid nephrosis.

Substance SC, according to Lowe *et al.* (1957), is a regular constituent of animal kidney. Human kidney with about 13 μg./g. is the richest source of SC yet known, with the exception of the liver of the vitamin A-deficient rat. From Table 5 it is seen that 14 out of 15 subjects with kidney disease exhibited no SC. In the 'normal' human kidney SC is not sharply localized in the medulla or the cortex.

If substance SC is equally available to all segments of the nephron it may possibly be concerned in maintaining the functioning nephron. Substance SC appears at the age of 1–2 years, when kidney function approaches that of the adult, and disappears in nephritis and nephrosis in which the nephron is affected.

The four cases of sub-acute chronic nephritis, in which the kidneys were large and white, showed (Tables 5 and 10) markedly elevated percentages of unsaponifiable matter, most of the increase being accounted for by a rise in cholesterol content.

Among the clinical manifestations of nephrosis are oedema, albuminuria, low metabolic rate, lipaemia and hypercholesterolaemia (Leiter, 1931*a*). There is often a rise in blood cholesterol, especially esterified cholesterol (Lichtenstein & Epstein, 1931). Histological study of nephritic kidneys shows lipid deposits made up mainly of cholesterol esters. Leiter (1931*b*) analysed kidneys from seven cases of glomerulonephritis for free and combined cholesterol. The highest figure for free cholesterol (0·297 %) was not very high but the esterified cholesterol went up to 0·294 %, ten times the normal figure.

It has been shown (Clainesborough, 1929; Heymann & Startzman, 1946) that nephrotic hyperlipaemia is due neither to increased intestinal absorption of cholesterol nor to excessive fat intake. It is more likely to arise from increased mobilization (Heymann, Matthews, Lemm, Olynyk, Salehar & Gilkey, 1954; Peters & Van Slyke, 1946 . There is often a rise in blood cholesterol which, according to Lichtenstein & Epstein (1931), is mostly in the ester portion, but according to other reports affects the free and esterified cholesterol equally (cf. Thomas, Rosenblum, Lander & Fisher, 1951). London, Sabella & Yamasaki (1951) found that the deuterium content of plasma cholesterol in subjects given D_2O increased more slowly in the nephrotic than in the non-nephrotic subject. Byers, Friedman & Rosenman (1954) and Sadhu & Mookerjee (1955*a*) did not find any marked rise in concentration of liver cholesterol in nephrotic rats, but the rate of cholesterol synthesis in the kidney increased considerably.

Lyman & Barron (1940), using rats with experimental nephritis, found notable depression of oxidative processes in kidney slices with succinate,

lactate, pyruvate and alanine as substrates. Sadhu & Mookerjee (1955*a*) suggest that in nephritis there is a partial blocking of the tricarboxylic acid cycle in the kidney tissue. Accumulation of acetate and acetoacetate might occur in the kidney and this might necessitate a diversion to cholesterol synthesis. The idea gains support from the observation that hypothyroid rats oxidize acetate at a reduced rate and synthesize more cholesterol (Sadhu & Mookerjee, 1955*b*).

The above considerations might well have a bearing on the problems of subacute chronic nephritis, but under the conditions of the present work the sterol contents of 'normal' kidneys and those characteristic of chronic nephritis and acute nephritis are very similar. It would have been attractive to correlate the disappearance of substance SC with an enhanced cholesterol synthesis, but the evidence does not justify it.

SUMMARY

1. The unsaponifiable matter from a variety of specimens of 'normal' human kidney has been studied. The percentage of unsaponifiable matter is about 0·33, of which about two-thirds is cholesterol. The quantitatively minor constituents include vitamin A (about 0·1 mg./100 g.), ubiquinone, substance SC and tocopherol, each about 1 mg./100 g. In all cases when the medulla and cortex were separated by dissection the tocopherol was found to occur only in the medulla. Kidneys from females were on the average richer in tocopherol than those from males.

2. Sixteen specimens of diseased kidney tissue have been studied. The ubiquinone content was similar to that found in 'normal' kidneys. The vitamin A content was not much changed. Tocopherol was not found in nephritic kidneys from females but nephritic kidneys from males contained normal amounts. Tocopherylquinone was often found in nephritic kidneys lacking tocopherol. It is possible that tocopherylquinone is an intermediate in the destruction of vitamin E.

3. Normal human kidney is (next to liver from vitamin A-deficient rats) the best source yet found for substance SC. This substance cannot be detected in diseased human kidneys.

4. In four cases of subacute chronic nephritis, in which the kidneys were large and pale, the percentages of unsaponifiable matter and in particular of cholesterol were much larger than in 'normal' kidneys. The change was much less marked in chronic nephritis and acute nephritis.

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Human Haptoglobins: Estimation and Purification

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The presence in mammalian sera of haptoglobins, a group of proteins characterized by their ability to bind haemoglobin, was established by Polonovski & Jayle (1939). Smithies (1955) demonstrated by starch-gel electrophoresis that, in humans, three types of haptoglobins can be distinguished in different individuals. Genetic control of the haptoglobin types by a pair of autosomal alleles, Hp^1 and Hp^2, has been established (Smithies & Walker, 1956). The homozygote Hp^1/Hp^1 shows a single haptoglobin component on electrophoresis, whereas the other homozygous type Hp^2/Hp^2 shows many components. The heterozygote Hp^2/Hp^1 also shows several haptoglobin components, but these in general differ in their electrophoretic mobilities from those of either of the homozygotes. The differences between the three genetic types thus appear to be complex, and an investigation of haptoglobins will be of considerable interest to biochemists and geneticists.

Part I. Estimation of haptoglobin

Two methods of estimating haptoglobin have been described. Jayle (1951) uses a method depending on the difference between the peroxidase activity of haemoglobin and its complexes with haptoglobin. Ethyl hydrogen peroxide is the oxidizing substrate, and iodide is the electron donor; iodine liberated during the reaction is determined by titration with thiosulphate. Laurell & Nyman (1957) titrate haptoglobin with haemoglobin, using filter-paper electrophoresis to determine the end point at which unbound haemoglobin first appears. The method of estimating haptoglobin here described depends on the peroxidase activity of haemoglobin–haptoglobin complexes, as does the method of Jayle (1951). Hydrogen peroxide is used as the oxidizing substrate. Guaiacol is the hydrogen donor (cf. Maehly & Chance, 1954). The reaction conditions are chosen so that the peroxidase activity of free haemoglobin is essentially zero. The formation of tetraguaiacol during the reaction is followed spectrophotometrically. The method is calibrated against a haemoglobin standard so that the haptoglobin content of a solution can be expressed in terms of its haemoglobin-binding capacity (cf. Laurell & Nyman, 1957).

METHODS

Reagents for standard estimation procedure

Hydrogen peroxide. A solution of hydrogen peroxide, 0·600±0·005 M, is prepared immediately before use by dilution (15- to 20-fold) of a stock solution. The dilute peroxide solution is discarded after 30 min. and if necessary a fresh dilution is prepared. The stock solution, approximately 30% (w/v) in hydrogen peroxide, is standardized at least once a week by titration with potassium permanganate (Schumb, Satterfield & Wentworth, 1955).

Guaiacol reagent. A buffered solution of guaiacol, 0·030±0·001 M, is prepared as follows: 1·86 g. of guaiacol (analytical grade) and 50 ml. of M-acetic acid are added to 400 ml. of water. The pH is carefully adjusted to 4·0 with aqueous sodium hydroxide, with a pH meter standardized with 0·05 M-potassium acid phthalate (pH 3·97 at 20°). The volume is made up to 500 ml. with water. The guaiacol reagent is stable for several weeks when stored in the cold.

Methaemoglobin solution. Wet crystals of human oxyhaemoglobin, crystallized by the procedure of Drabkin (1950), are dissolved in water. The solution is made to contain approximately 0·2 m-equiv. of haemoglobin/l. (i.e. 3·3 g./l.) on the basis of Drabkin's extinction coefficients for oxyhaemoglobin. An equal volume of 0·4 mM-potassium ferricyanide is added to convert the oxyhaemoglobin completely into methaemoglobin. The methaemoglobin solution is then carefully diluted to 0·050±0·004 mM. This concentration corresponds to the following extinction values calculated from Drabkin's data for methaemoglobin: 0·219 at 578 mμ; 0·218 at 562 mμ; 0·335 at 542 mμ. The solution is dispensed in small tubes and stored at −20°. It is stable for several months under these conditions.

Apparatus

A Beckman DU spectrophotometer is used to follow the peroxidase reaction. The temperature of the cell compartment is maintained at 30° with water circulating from a constant-temperature bath.

Standard estimation procedure

The three reagents are placed in a water bath at 30°. The solution to be tested is mixed with an equal volume of the methaemoglobin reagent. A portion (0·20 ml.) of the mixture is transferred to a cuvette of 1 cm. optical path and 2·75 ml. of the guaiacol reagent is added. The wavelength scale of the spectrophotometer is set at 470 mμ, the absorption maximum for tetraguaiacol, and the slit is opened to a convenient width (approximately 0·3 mm. with the DU spectrophotometer) so that the instrument can be operated at low sensitivity with the stable hydrogen lamp. The spectrophotometer is adjusted to give a reading of zero on the extinction scale with the cuvette containing the

sample in the optical path. The hydrogen peroxide reagent (0·05 ml.) is added from a polyethylene stirrer (a square of polyethylene sheet, 0·9 cm. × 0·9 cm., to which a polyethylene rod is attached centrally), which facilitates rapid addition of the hydrogen peroxide to the cuvette and thorough mixing. At the time of addition of peroxide a stopwatch is started. The extinction scale is set to successively higher values during the course of the peroxidase reaction, and the time at which the galvanometer needle passes zero is recorded for each extinction setting. The settings should be chosen so that, except for samples giving a very low peroxidase activity, the time between readings is of the order of 5–10 sec. A tape recorder is convenient for noting the times. Observations are continued until the rate slows appreciably.

The extinction readings ($\times 10^3$) are plotted against time (see Fig. 1). The slope in extinction units $\times 10^3$/sec. of the linear section of the progress curve is used as a measure of the peroxidase activity. The reaction shows a lag phase which is disregarded in calculating the slope. Samples giving a peroxidase activity of greater than 20 extinction units $\times 10^3$/sec. should be diluted and tested at a lower concentration. The haptoglobin content of the sample, in terms of its haemoglobin-binding capacity, is obtained from the measured peroxidase activity by reference to the calibration curve (Fig. 5).

RESULTS AND DISCUSSION

The method described above provides a rapid and accurate procedure for the estimation of haptoglobin in serum or in purified preparations. The reagents and specific reaction conditions for the standard estimation procedure were chosen on the basis of the following criteria.

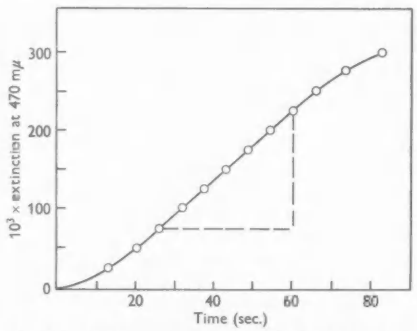


Fig. 1. A typical progress curve obtained during a haptoglobin estimation by the standard estimation procedure. The peroxidase reaction is followed by measuring the extinction at 470 mμ. The observed extinction ($\times 10^3$) is plotted against time (in sec.). The slope, in extinction units $\times 10^3$/sec., of the linear portion of the progress curve (indicated by the broken lines) is used as a measure of the peroxidase activity. The haptoglobin content of the test solution is obtained from this activity by reference to the calibration curve (Fig. 5).

Haemoglobin. Oxyhaemoglobin and methaemoglobin are closely similar in their peroxidase activities when combined with haptoglobin. However, methaemoglobin was selected for the standard estimation procedure because of its greater stability during storage. Although methaemoglobin prepared from crystalline oxyhaemoglobin has been used in the present work, methaemoglobin prepared directly from laked red cells would undoubtedly be satisfactory. The ferricyanide used in preparing the methaemoglobin does not interfere with the estimation of haptoglobin and it need not be removed.

Peroxide. Hydrogen peroxide and ethyl hydrogen peroxide are both satisfactory substrates for the haemoglobin–haptoglobin complex under the conditions described, but hydrogen peroxide is more convenient. The rate of the reaction is very sensitive to the concentration of peroxide (see Fig. 2). The concentration of hydrogen peroxide (0·01 M) for the standard estimation procedure was selected to give suitable reaction rates.

Guaiacol. Guaiacol was chosen as the reducing substrate because, at concentrations greater than 2·5 mM, it proved to be a powerful inhibitor of the peroxidase activity of free haemoglobin (Fig. 3). It also inhibits the activity of the haemoglobin-haptoglobin complexes, but to a much lesser extent. A guaiacol concentration of 27·5 mM in the reaction mixture permits advantageous use of this difference in inhibition.

Experiments with guaiacol at low concentrations, and with other hydrogen donors (e.g. leuco-Malachite green, *p*-toluidine), showed that human serum contains substances which inhibit the formation of the coloured products in the peroxidase reaction, presumably by competing with the added leuco dye. Guaiacol at 27·5 mM is sufficiently concentrated to overcome completely the inhibiting effect of serum. The other hydrogen donors tested are not sufficiently soluble to achieve this effect.

Effect of pH. Polonovski & Jayle (1939) observed that the pH optimum for the peroxidase activity of free haemoglobin is shifted to a more acid pH in the presence of haptoglobin. The pH optimum for free haemoglobin under the present conditions is 5·3, and the reaction rate diminishes rapidly as the solution is made more acidic (see Fig. 4). On the other hand, the peroxidase activity of the haemoglobin–haptoglobin complex increases on the acid side of pH 5·3, reaching its greatest value at pH 4·0. In still more acid solutions the rapid fading of tetraguaiacol prevents accurate measurement of the rate. Experiments with leuco-Malachite green as the hydrogen donor, instead of guaiacol, suggest that the true pH optimum for the complex is near pH 3·0. A pH of 4·0 was selected for the standard estimation procedure since at this pH the

peroxidase activity of free haemoglobin is negligible and the observable activity of its complexes with haptoglobin is nearly maximal.

These effects of pH on the peroxidase activity of haemoglobin and its complexes with haptoglobin suggest that haptoglobin protects haemoglobin from the denaturing effect of acid solutions. Thus, as the pH increases, the peroxidase activity is progressively less affected by the presence of haptoglobin.

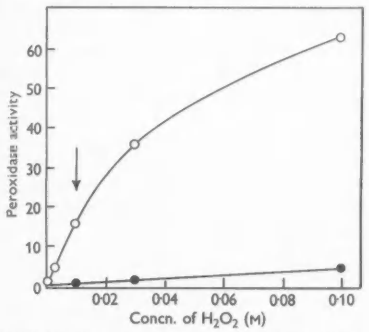


Fig. 2. Effect of hydrogen peroxide concentration on the peroxidase activity of methaemoglobin in the presence (○) and absence (●) of haptoglobin. Standard conditions were used for each determination except that the concentration of hydrogen peroxide was varied. The concentration of hydrogen peroxide selected for the standard estimation procedure is indicated by the arrow.

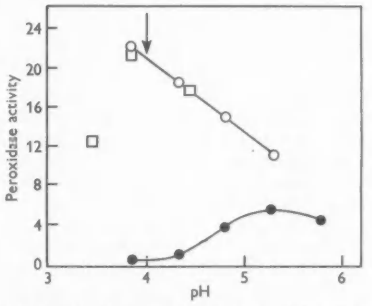


Fig. 4. Effect of pH on the peroxidase activity of methaemoglobin in the presence and absence of haptoglobin. ●, Methaemoglobin alone; ○, methaemoglobin plus haptoglobin, acetate-buffer solutions; □, methaemoglobin plus haptoglobin, lactate-buffer solutions. Standard conditions were used for each determination except that the pH and, where necessary, the buffering anion were varied. The pH selected for the standard estimation procedure is indicated by the arrow.

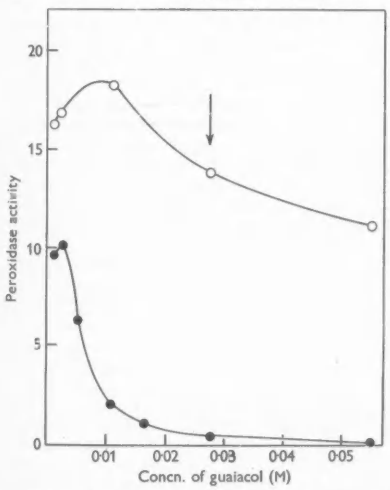


Fig. 3. Effect of guaiacol concentration on the peroxidase activity of methaemoglobin in the presence (○) and absence (●) of haptoglobin. Standard conditions were used for each determination except that the concentration of guaiacol was varied. The concentration of guaiacol selected for the standard estimation procedure is indicated by the arrow.

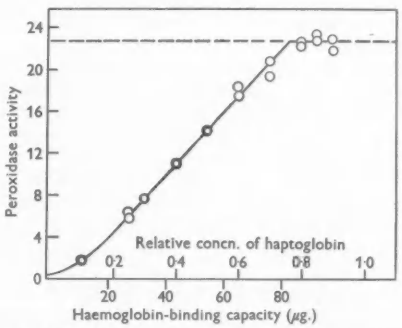


Fig. 5. Effect of increasing amounts of haptoglobin on the peroxidase activity of methaemoglobin measured by the standard estimation procedure. Coincident points are indicated by heavier circles. The haptoglobin solutions used in these experiments were obtained by diluting a solution of purified haptoglobin type 2-2. The upper scale on the abscissa indicates the relative concentration of haptoglobin. The haemoglobin-binding capacity of a given haptoglobin solution can be obtained from its peroxidase activity, measured by the standard estimation procedure, by reference to the lower scale on the abscissa (see text, Part I, Calibration of the method). The horizontal broken line indicates the peroxidase activity of 82·5 μg. of methaemoglobin when fully saturated with haptoglobin (see text).

Calibration of the method. A series of haptoglobin solutions of increasing dilution was prepared from a purified haptoglobin preparation (type 2-2), and the peroxidase activity was determined for each solution by the standard estimation procedure, but with a more accurately standardized haemoglobin solution (0·05 ± 0·0005 mM) than is normally used. In Fig. 5 the observed activity is plotted against the relative concentration of haptoglobin. Curves identical within experimental error have been obtained with haptoglobins of the other two common types. The curve rises initially and then becomes linear. At high haptoglobin concentrations there is a point of inflexion and the curve becomes parallel to the abscissa. The maximum peroxidase activity obtained, corresponding to this horizontal part of the curve, was found to be independent of the type of haptoglobin used. A mean value of 22·5 ± 0·55 extinction units × 10^3/sec. was obtained for ten samples of haptoglobin, including all three common types (see broken line in Fig. 5).

The meaning of the point of inflexion was established by preparing a similar curve, with only half the standard amount of methaemoglobin being used in the reaction mixture. This curve reached a plateau at approximately half the former maximum activity value. Thus at the point of inflexion all of the added methaemoglobin is present as haptoglobin complex, and further addition of free haptoglobin does not increase the peroxidase activity. This interpretation is confirmed by the results of a starch-gel electrophoresis experiment which showed that the solutions with activities corresponding to the plateau contained no free methaemoglobin.

Since 82·5 μg. of methaemoglobin (0·1 ml. of 0·05 mM-solution) is used in the standard estimation procedure, the amount of haptoglobin corresponding to the inflexion point has a haemoglobin-binding capacity of 82·5 μg. With this point of reference, the lower scale of the abscissa of Fig. 5 was laid out, expressing the amount of haptoglobin in the reaction mixture in terms of its haemoglobin-binding capacity. Thus in order to determine the haptoglobin content of a given solution the peroxidase activity of the solution is first measured by the standard estimation procedure. The haptoglobin content expressed in terms of a haemoglobin-binding capacity is then read from the curve in Fig. 5. Numerically the haemoglobin-binding capacity of the test solution (in μg.) is the same as the amount of haemoglobin (in mg.) which would be bound by 100 ml. of the same solution.

It seems desirable to express amounts of haptoglobin in terms of haemoglobin-binding equivalents because the function of haptoglobin is thought to be related to its ability to bind haemoglobin and to prevent the loss in the urine of haemoglobin liberated into the blood stream (Allison & ap Rees, 1957; Laurell & Nyman, 1957). Furthermore, since haemoglobin solutions of known concentration can be made accurately, haptoglobin determinations in different laboratories can be directly compared, provided that a calibration curve is prepared.

The method described can be applied equally well to serum and to purified preparations of haptoglobin. If a sample of purified haptoglobin is added to serum, the haptoglobin content of the mixture determined by the standard estimation procedure is equal, within experimental error, to the sum of the haptoglobin contents of the original serum and of the purified haptoglobin preparation. Thus the constituents of normal serum do not interfere with the estimation.

Part II. Purification of haptoglobin

Jayle, Boussier & Tonnelat (1956) and Boussier (1958) have described methods of purifying haptoglobins from serum by salt-fractionation and preparative electrophoresis. The present paper describes a procedure involving a single stage of adsorption and desorption by which haptoglobins of high purity uncombined with haemoglobin can be prepared from serum with 40–50 % yields.

METHODS

Dowex 2 X-10 (200–400 mesh) anionic-exchange resin*, as the chloride, is used to adsorb the haptoglobins from dialysed serum at pH 4·2. The resin is set up in a column after the adsorption and is washed with water. The haptoglobins are eluted with 0·05 M-NaCl. All steps are carried out at room temperature unless otherwise stated.

Preparation of serum. Small samples of serum were prepared directly from clotted whole blood with precautions to avoid haemolysis. Larger samples were obtained from blood-bank acid–citrate–dextrose (A.C.D.) blood, only bottles in which the plasma appeared to be essentially free from haemolysis being used. The plasma was separated by centrifuging and 3 M-calcium chloride was added in amounts equivalent to the citrate present. The plasma was allowed to clot in centrifuge tubes overnight, and the serum was obtained by centrifuging at 25 000 *g* for 30 min. in a refrigerated centrifuge. Sufficient M-disodium citrate was added to combine with the Ca^{2+} added previously, and the serum was stored at −20° until required.

Adsorption to resin. Sodium acetate buffer is prepared (0·2 M in acetic acid and 0·04 M in sodium hydroxide). The pH is carefully adjusted to 4·2, with a pH meter standardized with 0·05 M-potassium acid phthalate (pH 3·97 at 20°). Serum is mixed with 3 vol. of this buffer and the resulting solution is dialysed against 10 times its volume of water acidified to pH 4·2 with hydrochloric acid. The dialysis is carried out in the cold room with stirring, and the external

* *Note added in proof*: Dowex 2 is not now available in the fine-mesh grade. Dowex 1 X-8 (200–400 mesh) appears to be an adequate substitute.

liquid is changed five times at 12 hr. intervals. Thorough dialysis is essential. The final pH should be between 4·2 and 4·7.

The resin is prepared by washing twice in a column with M-sodium hydroxide solution, water, M-hydrochloric acid and water. The last washing with water should be very thorough. One volume of packed resin in water with 0·4 vol. of dialysed serum and 0·6 vol. of water are mixed in a beaker, stirred for 15 min. and then poured into a column for washing and elution. The resin column is washed with water until the extinction of the effluent falls below 0·02 at 280 mμ. 0·05M-Sodium chloride solution is layered on to the top of the column and elution under gravity flow is carried out. A convenient pressure head is obtained when the height of the liquid above the top of the resin is approximately one-fifth of the length of the resin column. The appearance of the sodium chloride front is detected by a simple conductance tip on the outflow of the column (see Fig. 6). Collection of the effluent is commenced as soon as the conductivity begins to increase. The haptoglobin is obtained in the first 2–80 ml. of effluent with applied volumes of dialysed serum of 12–600 ml. Serial fractions are analysed for haptoglobin by the method described above, and for total protein by measuring the extinction at 280 mμ. The maximum concentration of haptoglobin obtained is between two and four times the initial concentration in the undiluted serum. Preparations have been carried out with as little as 1 ml. and as much as 150 ml. of undiluted serum. The yields and purity of the haptoglobin are independent of the amounts of serum used over this range. The resin can be regenerated by treatment with 2M-HCl at 100° for 24 hr. followed by exhaustive washing. Treatment with alkali is unnecessary.

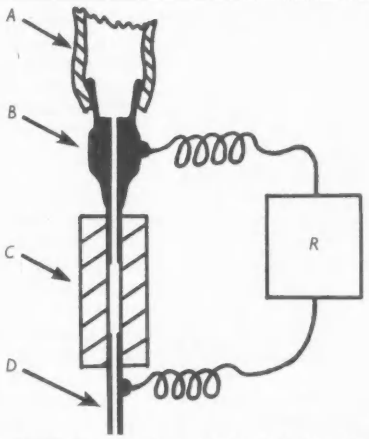


Fig. 6. A simple conductance tip for following the conductivity of column effluents. *A*, Rubber tube connected to outflow of column; *B*, 16-gauge hypodermic needle forming upper electrode of conductance cell; *C*, Perspex block; *D*, part of 16-gauge hypodermic needle forming lower electrode of conductance cell. Needle electrodes *B* and *D* are tightly fitted into a hole drilled through *C*. *R* is a simple resistance meter (model 630, Triplett Electrical Instrument Co., Bluffton, Ohio, U.S.A.).

RESULTS

Table 1 summarizes the results of a haptoglobin preparation from the serum of an individual of haptoglobin type 2-1. In this preparation the yield of purified haptoglobin from the column amounted to 48 % of the haptoglobin present in the starting material. The total recovery (column washings and purified fractions) was 103 % of the haptoglobin of the starting material, so that no inactivation occurred during the purification procedure. The highest specific activity obtained in any fraction of this preparation was 42·7 mg. of haemoglobin-binding capacity/100 ml./$E_{280\ m\mu}$. In several preparations the specific activity of the richest fraction averaged 46·0. Preliminary studies in the ultracentrifuge and by electrophoresis in starch

Table 1. *A typical preparation of purified haptoglobin*

A.C.D. serum (150 ml.), haptoglobin type 2-1, was subjected to the purification procedure described under Methods. Fractions (10 ml.; 1–8) from the column were analysed for haptoglobin content by the standard estimation procedure, and for total protein by measuring the extinction at 280 mμ. Fractions 3 and 4, marked with asterisks, contained so much haptoglobin that their haptoglobin contents were determined on fivefold diluted samples; the figures in the corresponding rows of the table are, however, presented as if the dilutions had not been made. Hb, haemoglobin.

Fraction	$E_{280\ m\mu}$	Slope of progress curve (10^3 E/sec.)	Activity (mg. of Hb-binding capacity/100 ml.)	Specific activity (activity/E)	Total Hb-binding capacity (mg./fraction)
Starting material (diluted dialysed serum)	13·5	3·57	18·1	1·34	108·6
Column washings	4·0	0·57	2·9	0·72	58·0
1	0·02	0	0	—	0
2	1·68	19·3	71·7	42·7	7·2
3*	4·77	49·0	199	41·7	19·9
4*	3·42	29·7	134	39·1	13·4
5	1·76	19·6	72·5	41·2	7·3
6	0·94	8·9	36·7	39·0	3·7
7	0·57	3·7	18·7	33·0	1·9
8	0·33	1·6	8·8	26·4	0·9

gels, of typical preparations of purified type 1-1 haptoglobin, suggest that the best preparations obtained are approximately 80 % pure.

Purified haptoglobins were prepared from sera of the three common haptoglobin types, Hp 1-1, Hp 2-1 and Hp 2-2, and from serum of a fourth type, Hp 2-1 (Mod.), which is rarely seen in whites, but occurs comparatively frequently in negroes (approximately one in ten). This fourth type is referred to as Hp 2-1 (Mod.) because it appears to be a modified form of haptoglobin type 2-1; the rates of migration in starch gel of the individual type 2-1 (Mod.) haptoglobins are the same as those of the type 2-1 haptoglobins, but the relative concentrations of the individual components are different. A photograph of the results of a vertical starch-gel-electrophoresis experiment (Smithies, 1959) with these four preparations is presented in Plate 2, which shows the portion of the gel from the sample slots to the fastest migrating haptoglobin. All the components which are clearly visible in the photograph have been identified as haptoglobins, by showing that they will combine with added haemoglobin. When complexed with haemoglobin the individual haptoglobin components migrate in starch gel less rapidly than in the absence of haemoglobin, but the general appearance of the patterns for the four genetic types is otherwise unchanged.

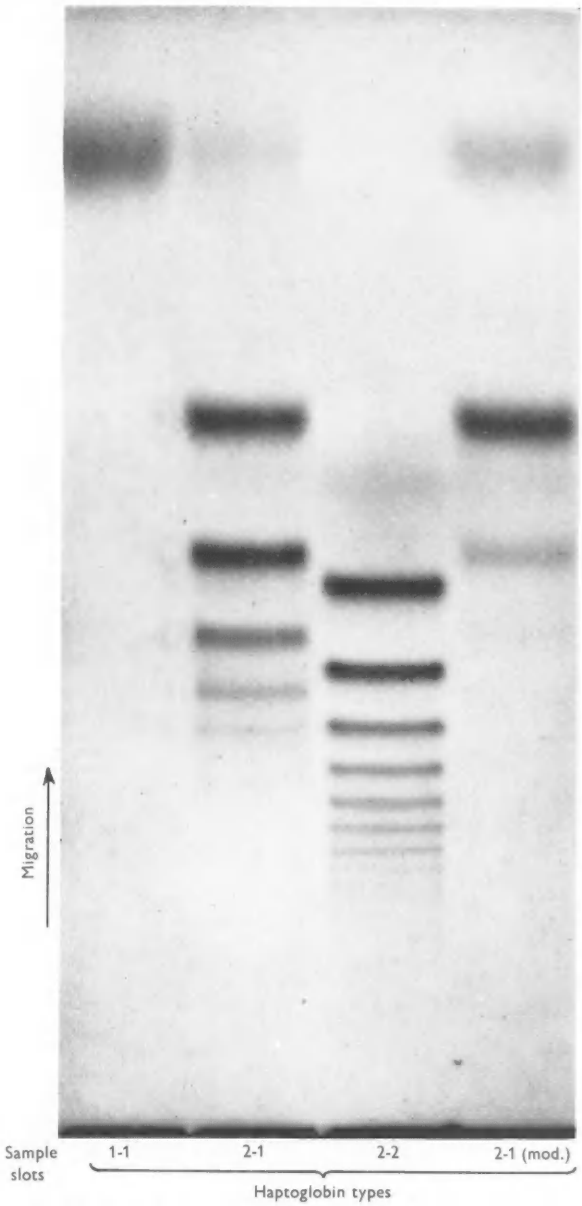
DISCUSSION

The absorption of haptoglobin at pH 4·2 by the positively charged Dowex 2 resin suggests that haptoglobin is a protein with a low isoelectric point. Most serum proteins at this pH have a net positive charge. The method for the preparation thus appears to depend on the selection of a pH at which haptoglobin is the chief serum protein with a net negative charge. This hypothesis receives confirmation from the observation that haptoglobin of progressively greater purity was obtained as the pH of adsorption was decreased from 5·5 to 4·3. Adsorptions below pH 3·9 yielded no haptoglobin. Rabbit haptoglobin has been successfully prepared by the procedure here applied to human haptoglobins, which suggests that haptoglobins in species other than man may be acidic proteins.

Haptoglobins purified by the present procedure are of value for further investigation of their properties only in so far as the haptoglobins are undamaged by the purification. Evidence bearing on this question is provided by the experiment illustrated in Table 1, in which the haptoglobin content of the serum was completely accounted for in the column washings and the purified fractions. No significant loss of haemoglobin-binding capacity occurred. Further evidence is provided by the results illustrated in Plate 2, which show that there are at least seven components in purified haptoglobin of type 2-1 and more than 12 in purified haptoglobin of type 2-2. The number of components seen in these preparations is thus closely similar to the number which can be demonstrated in whole sera of the same genetic type and of equivalent haptoglobin content (cf. Smithies, 1959, serum samples 1 and 2). Furthermore the behaviour in starch-gel electrophoresis of the purified haptoglobins of the several genetic types, both with respect to the mobility and the relative amounts of the different components, is the same as the behaviour of the respective haptoglobins in the sera from which they were obtained. This is particularly well illustrated by the preparation of haptoglobin type 2-1 (Mod.) illustrated in Plate 2. The principal difference between this type and haptoglobin type 2-1 is in the relative amounts of protein in the different haptoglobin zones. The relative proportions which were observed in the original serum of this type were completely preserved in the purified preparation. All the purified haptoglobins retain their ability to bind haemoglobin.

Franglen & Gosselin (1958) described a simple experiment in which a quininoid dye was separated into two components by starch-gel electrophoresis. They established that these two components were in dynamic equilibrium, since either component separated again into two bands when it was isolated from the gel and subjected to a second electrophoresis. It is thus of great importance to establish that the large number of haptoglobin zones are not artifacts of the starch-gel electrophoretic method and that they represent 'real' protein components.

Two approaches to this problem were employed. Serum of haptoglobin type 2-2 was subjected to starch-gel electrophoresis. The regions of the gel containing the main haptoglobin zones were cut out, frozen, thawed and the proteins were obtained in solution by centrifuging (cf. Smithies, 1955). These haptoglobin solutions were kept at 37° for 20 hr., and were subjected to a second electrophoresis alongside the original serum. The individual haptoglobins retained their original electrophoretic characteristics. Storage for several days at 5° of individual haemoglobin–haptoglobin complexes similarly isolated from the gels was without effect on their electrophoretic properties. Further strong evidence for the reality of the many haptoglobin zones in the purified preparations was obtained by observing the behaviour of the preparations during starch-gel electrophoresis under a variety of conditions. The characteristic starch-gel patterns for the three common haptoglobin types were obtained at pH 9·0 in the presence of 8 M-urea, at pH 8·9 in ethylenediaminetetra-acetic acid buffer (0·01 M) and at pH 8·5 with barbiturate buffer of the ionic strength usual for filter-paper electrophoresis.



A photograph of the results of a starch-gel-electrophoresis experiment with four preparations of purified haptoglobin of the genetic types indicated. The portion of gel from the sample slots to the fastest-migrating haptoglobin is shown. The gel was stained with Amido-Black 10B.

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(*Facing p.* 120)

Similar patterns, but with mobilities of reversed sign, were obtained at pH 2·1 and 1·7 in dilute hydrochloric acid gels. These results strongly suggest that the many haptoglobin zones demonstrated under the usual conditions of starch-gel electrophoresis, in whole sera and in the purified preparations, are not artifacts but represent distinct protein components.

SUMMARY

1. A procedure for the rapid, accurate estimation of haptoglobin in serum and purified preparations is described, based on the peroxidase activity of haemoglobin in its complexes with haptoglobin.

2. Hydrogen peroxide, guaiacol and methaemoglobin reagents are used, under well-defined conditions which reduce the peroxidase activity of free haemoglobin essentially to zero. The reaction is followed spectrophotometrically.

3. The method is calibrated so that the haptoglobin content of a solution can be expressed in terms of its haemoglobin-binding capacity.

4. Haptoglobin has been prepared in high purity from 1 to 150 ml. of serum in approximately 50% yield, by bulk-adsorption from dialysed serum at pH 4·2 with Dowex 2 X-10 anionic-exchange resin in the chloride form, followed by washing in a column and elution with 0·05 M-sodium chloride solution.

5. Purified haptoglobins from sera of the three common haptoglobin types and from serum of the modified type, Hp 2-1 (Mod.), have been examined by starch-gel electrophoresis.

6. Evidence is presented that the haptoglobins obtained are undamaged by the purification, and that the many individual haptoglobin components demonstrated in the several genetic types are not artifacts.

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The Genetic Control of Transferrins in Humans

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Inherited differences in the serum proteins of normal individuals which are apparently unassociated with red-cell blood groups have been recently described. Smithies (1955) and Smithies & Walker (1956) reported evidence for the genetic control of serum haptoglobins; Grubb (1956) and Grubb & Laurell (1956) showed differences in the serum γ-globulins of different persons and demonstrated the genetic control of these differences. This paper describes the experimental procedures used and the family studies made to establish the existence of a third genetic system involving the serum proteins of normal individuals.

Smithies (1957) observed in the sera of two New York negroes (out of 50 studied) and five Australian aborigines (out of 23 studied) the presence of an additional β-globulin (D), not previously described. Family studies, reported by Horsfall & Smithies (1958), indicated that the presence or absence of β-globulins C and D in the serum was under genetic control. Three phenotypes were observed in the Australian aborigines corresponding to the possible combinations of two alleles at a single autosomal (i.e. not sex-linked) locus. In searching for the occurrence of β-globulin D in the sera of Canadian whites, Smithies (1958) found five

individuals (out of 425 studied) in whose sera β-globulins were present which suggested that a third allele was possible at the same locus. Family studies, initiated at that time and now completed, confirm the involvement of this third allele.

Variations in serum β-globulins are not confined to humans. Similar variations in the sera of cattle have been described by Hickman & Smithies (1957), who suggested that three autosomal alleles were involved in the genetic control of the five types of cattle β-globulins which they observed. Ashton (1957) independently described the same five types and suggested that five linked genes might be involved in their genetic control. The detailed reports of these workers (Smithies & Hickman, 1958, and Ashton, 1958) indicate that all the data are consistent with a three-allele hypothesis, when incorrectly recorded matings are excluded. Ashton (1958) reports seeing the sixth of the possible phenotypes from three alleles, in addition to the five which were reported in both initial accounts. In humans four phenotypes have been observed and two more are postulated (see below).

The proteins involved in the genetically controlled variations of the serum β-globulins in humans have been identified as transferrins (see Discussion), and the corresponding types will therefore be referred to as transferrin types in the following account.

EXPERIMENTAL METHODS

The starch-gel electrophoretic methods described by Smithies (1955, 1959) and Poulik & Smithies (1958) have been used in this work, with suitable modifications of the two-dimensional method to permit the testing of several sera at once.

The two-dimensional method proved most reliable for the determination of transferrin types, particularly when the occurrence of transferrin D in a serum sample was under investigation. [However, Harris, Robson & Siniscalco (1958) find that the determinations can be made by one-dimensional starch-gel electrophoresis, if the discontinuous buffer system described by Poulik (1957) is used and excess of haemoglobin is added to the serum samples.] Usually 15 sera were tested by the two-dimensional method in the following way.

Fifteen strips (1·0 cm. × 25 cm.) of Schleicher and Schüll 598 YD filter paper were set up in the moist chamber with the usual buffer solution (see Poulik & Smithies, 1958). A volume (0·015 ml.) of serum, to which haemoglobin had been deliberately added (4 drops of serum to 1 drop of haemoglobin solution, approximately 3 g. of haemoglobin/100 ml.), was applied to each strip. Electrophoresis was carried out overnight (16–18 hr.) with 110 v across the electrodes.

In the morning three starch gels (12 cm. × 16 cm. × 0·6 cm.) were prepared in the usual way. A cut was made along the 16 cm. length of each gel approximately 1 cm. from the edge of the tray, and the 1 cm. strip of gel was removed. A second cut was made approximately 3 cm. from the first cut, and the smaller part of the gel was displaced to open up a slot in the gel. A 3 cm. length was then cut from each filter-paper strip so as to include the region over which the β-globulins were distributed, the position of the free haemoglobin band being used as a guide to the correct region (haemoglobin not bound to haptoglobin migrates at the same rate as the β-globulins in this filter-paper-electrophoresis system). The cutting and handling of these 3 cm. strips during their insertion into the gels was helped by using a plastic spring clip to hold the edge of the filter paper. The 1 cm. wide strips protruded above the top of the 0·6 cm. thick gels so that the clip did not interfere with the insertion. When five pieces of filter paper had been inserted into a gel, the displaced gel portion was returned to its original position and the 1 cm. piece of gel was replaced. Petroleum jelly (just warm enough to be liquid) was poured over the gel and protruding pieces of filter paper, except for a 2 cm. wide area at the ends of the gels. Electrical contact was made to these uncoated areas with thick wads of filter paper soaked in bridge solution, and electrophoresis was carried out for 5–6 hr. at 6 v/cm. The gels were then removed from the trays and sliced and stained in the usual way (Smithies, 1955).

The rates of migration of transferrins B and C differ only slightly so that detection of transferrin B by two-dimensional electrophoresis was replaced in later stages of the work by one-dimensional electrophoresis for approximately three times the usual time (see Smithies, 1959). The longer period one-dimensional experiments proved perfectly satisfactory for the detection of transferrins B and C in sera of all haptoglobin types provided that the sera were relatively free from haemolysis. The one-dimensional experiments were not so satisfactory for the detection of transferrin D (but see Harris *et al.* 1958).

RESULTS

Electrophoretic studies

Fig. 1 shows diagrammatically the results of two-dimensional starch-gel electrophoresis experiments with sera, plus added haemoglobin, of the four transferrin types which have so far been observed (Tf BC, Tf CC, Tf CD and Tf DD). A mixture of equal amounts of sera of transferrin types Tf CC and Tf DD gives a result indistinguishable, as far as the transferrins are concerned, from that obtained with a serum of type Tf CD.

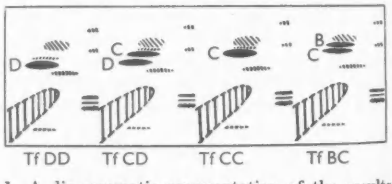

Fig. 1. A diagrammatic representation of the results of two-dimensional starch-gel electrophoresis experiments with the β-globulins from sera of the four transferrin types so far observed. Left to right: Tf DD, Tf CD, Tf CC and Tf BC. The transferrins (B, C and D) are lettered in the diagram.

Fig. 2 shows a photograph of a one-dimensional starch-gel experiment with the vertical technique described by Smithies (1959), using sera of the same four transferrin types, again in the presence of added haemoglobin. The haptoglobin types of these individuals are such (Hp 2-2) that transferrin D can be demonstrated by one-dimensional electrophoresis. Rather unusual post-albumins (see Smithies, 1959) are present in the two sera on the left-hand side of the gel. These sera are from a father and one of his sons; another of his sons did not show this unusual post-albumin pattern. The two sera on the right-hand side of the gel are from a father and his daughter.

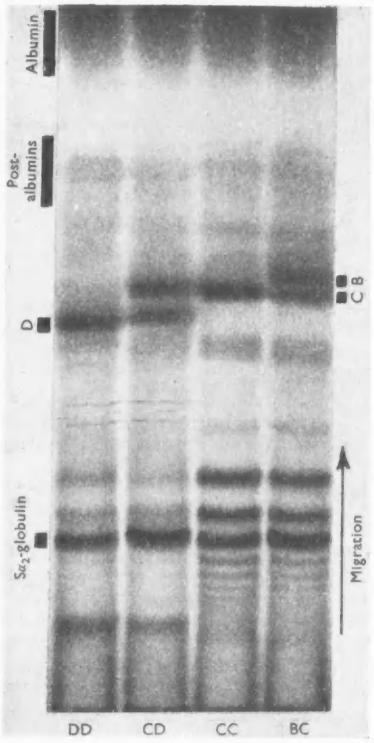

Fig. 2. A photograph of the results of a vertical starch-gel-electrophoresis experiment with sera (plus haemoglobin) of the transferrin types (left to right) Tf DD, Tf CD, Tf CC and Tf BC. All four sera are of haptoglobin type 2-2. The portion of the gel from the origin to the albumin is included in the photograph. The transferrins are lettered, and the positions of the albumin, post-albumins and Sz_2-globulin (Smithies, 1955) are indicated alongside the photograph.

Family studies

Figs. 3 and 4 show two typical family trees, from Australian aborigine and Scottish–Canadian families, in which occur the four transferrin types so far observed. Table 1 shows a summary of the family studies, presented in the form of a comparison of the observed and expected (from the three-allele hypothesis here considered) distributions of the transferrin types in 79 offspring of 28

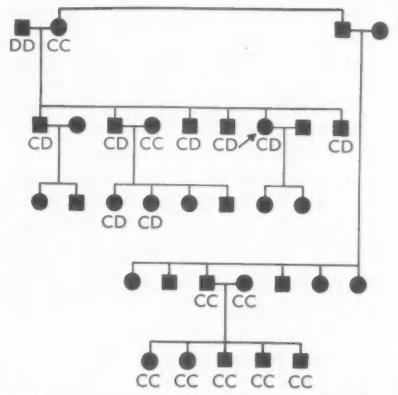

Fig. 3. The pedigree of an Australian aborigine family in which the three transferrin types Tf CC, Tf CD and Tf DD were observed. The letters under the symbols (■, male; ●, female) indicate the transferrin types. The proband is shown with an arrow.

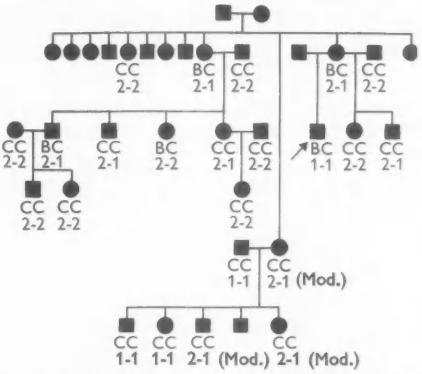

Fig. 4. The pedigree of a Scottish–Canadian family. The transferrin types are shown by the letters. The numerals indicate the haptoglobin types. Independent segregation of the genes at the *Tf* and *Hp* loci is seen in this family (■, male; ●, female), which also illustrates the inheritance of the modified haptoglobin type, Hp 2-1 (Mod.). The proband is shown with an arrow.

Table 1. *Distributions of transferrin types in the 79 offspring of 28 matings*

Combined data of family studies made to establish the mode of inheritance of the transferrins. Twenty-eight complete matings have been seen (four types of mating). The observed distribution of transferrin types in the 79 offspring is compared with that expected from the three-allele hypothesis here considered. Transferrin types Tf BB and Tf BD have not yet been observed. The results are given as observed/expected distributions, e.g. 0/0.

Observed matings	No. of matings	Offspring transferrin types					
		(BB)	BC	(BD)	CC	CD	DD
BC × CC	9	0/0	14/13·5	0/0	13/13·5	0/0	0/0
CC × CC	12	0/0	0/0	0/0	28/28·0	0/0	0/0
CC × CD	6	0/0	0/0	0/0	9/9·0	9/9·0	0/0
CC × DD	1	0/0	0/0	0/0	0/0	6/6·0	0/0

matings, in which both parents and at least one child were tested. Offspring of many more matings between individuals having only transferrin C in their sera have been observed, but only results of those matings between individuals related to persons showing either transferrins B or D are presented here. The table includes the data reported by Horsfall & Smithies (1958) in their account of studies on Australian aboriginal families; Fig. 3 is the pedigree of one of these families. The remaining data were collected during studies made of the available families of the five Toronto blood donors in whose sera transferrin B was first observed (Smithies, 1958), together with one family encountered in a survey of the families of diabetic individuals.

DISCUSSION

Identity of transferrin and the genetically controlled β-globulins

Three independent lines of evidence are given below that β-globulin C (the common β-globulin in all populations so far studied) is, in fact, transferrin. The genetic studies here reported suggest that the less-common β-globulins B and D are controlled by genes at the same locus as that controlling β-globulin C. Consequently these three genetically controlled β-globulins are presumed to be transferrins (iron-binding proteins, or siderophilins).

(1) From the average value for the iron-binding capacity of serum in normal individuals, the molecular weight of transferrin and the fact that each molecule of transferrin can bind 2 atoms of iron, the average transferrin content of normal sera can be calculated to be 0·25 g./100 ml. Transferrin migrates as a β-globulin during filter-paper electrophoresis (see Laurell, 1952, for a review and bibliography on transferrin). The only β-globulin demonstrable by two-dimensional starch-gel electrophoresis which is present in normal sera of the common type in sufficient quantities to correspond to transferrin is β-globulin C. Dr James H. Pert (personal communication) has made a provisional estimate, in confirmation of this, that the average amount of β-globulin C in normal sera is 0·25 ± 0·03 g./100 ml. Pert used a photoelectric reflectometer to scan the gels, and assumed for this purpose that the binding of dye by β-globulin C does not differ significantly from the average of the binding of dye by all the serum proteins.

(2) Mr M. D. Poulik (personal communication) has compared the rate of migration in starch gel of purified transferrin (prepared by Inman, 1956, from Cohn fraction IV-7 of pooled plasma) with that of β-globulin C. The rates are identical. The migrations of both purified transferrin and β-globulin C are altered in the same characteristic way by the presence of certain diphtheria toxins (cf. Poulik, 1956). Furthermore, β-globulin C reacts specifically with antisera prepared in rabbits against the purified transferrin, when tested by the starch-gel-immunoelectrophoretic method of Poulik (1958).

(3) Dr A. C. Allison (personal communication) finds that radioactive iron (^{59}Fe) given in small amounts orally (or added to serum *in vitro*) appears to be almost entirely bound to β-globulin C when the resulting sera are investigated by one- or two-dimensional starch-gel electrophoresis. Drs H. Eldon Sutton and Ronald C. Bishop (personal communication) added small amounts of ^{59}Fe to sera of the transferrin types Tf CC and Tf CD and observed that the resulting radioactivity was associated with β-globulin C in the Tf CC serum and approximately equally with both β-globulins C and D in the Tf CD serum. This direct observation of iron-binding by both β-globulins C and D confirms the presumption, made on the basis of genetic evidence, that β-globulin D is also a transferrin.

Possible nature of the differences in the transferrins

The differences between the transferrins B, C and D appear to be more simple than those between the haptoglobins. The results shown diagrammatically in Fig. 1 indicate that transferrin D migrates less rapidly than transferrin C, and the latter less

rapidly than transferrin B, in both the filter-paper and starch-gel-electrophoretic systems. This suggests that they carry different charges. It is thus not unlikely that the different transferrins will prove to differ by one, or at most a few, amino acid residues, as has been proved with some of the haemoglobins.

Genetic studies

The illustrative pedigrees shown in Figs. 3 and 4, and the summary of data presented in Table 1, suggest strongly that the transferrins are controlled at a single autosomal locus by at least three alleles. The symbol *Tf* is suggested for the locus; the alleles so far characterized can be designated Tf^B, Tf^C and Tf^D. Tf^C is the common allele in all populations so far sampled. Tf^B occurs approximately 1 in 100 in Canadian whites. Tf^D occurs approximately 1 in 25 in New York negroes and approximately 1 in 10 in Australian aborigines, but has not been observed in over 500 whites tested for their transferrin types. Racial differences in the frequencies of these alleles are thus indicated.

There are six phenotypes possible in the transferrin system if three alleles are involved. Two of these, Tf BB and Tf BD, have not been observed. Tf BB, in which transferrin B is present but both transferrins C and D are absent, should occur approximately 1 in 10 000 in Canadian whites. The frequency of occurrence of Tf BD, in which transferrins B and D are present in equal amounts, is difficult to estimate since it may depend on the frequency of inter-racial matings, as the alleles Tf^B and Tf^D have not yet been found in the same race.

The genetic control of the transferrins may prove to be more complex than is suggested by the family studies here presented. Thus Harris *et al.* (1958) have observed two persons in whose sera β-globulins were detected which appeared in the same general position in the gels as transferrin B. However, the β-globulin B in one of these persons migrated slightly less rapidly than that in the other. These workers also observed a similar situation with respect to β-globulin D. It is therefore possible that the transferrin types will need to be subdivided. We were unable to detect any significant differences in the rates of migration of transferrin B in the sera of representative members, all haptoglobin type 2-1, of the seven unrelated families in which this transferrin was observed, nor was any difference detectable in the rates of migration of transferrin D from the New York negroes and the Australian aborigines. The transferrins B and D described in this paper migrate in positions which apparently correspond to the β-globulins B_2 and D_1 respectively of Harris *et al.* (1958).

Independent segregation of the genes at the *Hp* (haptoglobin) and *Tf* loci is seen in the family tree shown in Fig. 4, and other examples of this segregation have been observed. This Scottish–Canadian family also shows the modified form (rare in whites) of the heterozygous haptoglobin type, Hp 2-1 (Mod.), described by Connell & Smithies (1959). The pedigree suggests that either a dominant modifier gene is involved in the genetic control of this modified haptoglobin type, or that there can be a third allele at the *Hp* locus, similar to Hp^2 but not identical with it. A recessive modifier gene is unlikely to be involved since this would require the husband, of the woman here showing this type and passing it on to two of her children, to be heterozygous for the recessive gene. This situation is very improbable since this woman (and her children) are the only white persons in whom this phenotype has been seen. No evidence of negro ancestry could be obtained from the red-cell blood groups of the members of this family, and they had very fair skins.

The red-cell blood groups of most of the individuals of the families in which the allele Tf^B was seen have been kindly determined for the author by Dr Bruce Chown. Independent segregation has been observed of the genes at the transferrin locus and those at the ABO, MNS, P and Rh loci. No correlation between the transferrin types and the Le, Fy, and Kell red-cell blood groups was found, although independent segregation of the genes at the transferrin locus and the genes at the loci corresponding to these three red-cell blood group systems was not observed.

SUMMARY

1. The methods used to demonstrate variations in the serum β-globulins of normal individuals are described.

2. The variable β-globulins are identified as transferrins.

3. Family studies (28 matings, 79 offspring) suggest that the transferrins are genetically controlled at a single locus, *Tf*, by at least three alleles, Tf^B, Tf^C and Tf^D.

4. Tf^C is the common allele. Tf^B occurs approximately 1 in 100 in Canadian whites. Tf^D occurs approximately 1 in 25 in New York negroes and approximately 1 in 10 in Australian aborigines.

5. Four of the six possible phenotypes have been observed: Tf BC, Tf CC, Tf CD and Tf DD.

6. Independent segregation of the genes at the *Tf* locus and those at the ABO, MNS, P, Rh and Hp loci has been observed.

The author is very grateful to Dr W. R. Horsfall for collecting the sera and compiling the pedigree of the Australian aboriginal family.

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Distribution of Sodium and Potassium within Cattle Lens

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In the lens there is no shedding or extrusion of old cells. It continues to grow, at a decreasing rate, throughout life, the fresh fibres being formed at the peripheral surface of the cortex and the older fibres becoming ever more compressed in the centre or so-called nucleus of the lens. Immediately beneath the anterior surface of the capsule which surrounds the lens is a single layer of epithelial cells, some of which undergo mitosis to provide the newly formed lens fibres. The lens therefore contains concentric zones of cells representing widely differing ages.

The sodium and potassium contents of cattle lens were measured by Fischer (1933); the aqueous humour and vitreous humour had already been analysed by Tron (1926, 1927). If these authors' results are converted into millimolal units, for purposes of comparison, we obtain 30·9 m-molal sodium and 160 m-molal potassium in the lens, compared with 148 m-molal sodium and 4·9 m-molal potassium in the aqueous and vitreous humours. The sodium and potassium content of the lens is therefore almost the inverse of that of the two humours.

Considering the peculiar growth characteristics of the lens, and the nature of its ionic environment, it would be interesting to know whether there are any differences between concentric layers of the lens in their contents of sodium and potassium. There seems to be only one reference in the literature to the distribution of these ions within the lens. Lebensohn (1936) gave the following values for normal adult ox lens (expressed in millimolal units): cortex, sodium 54·5 and potassium 90·1; nucleus, sodium 90·0 and potassium 120·6. This indicated a higher concentration of sodium in the nucleus than in the cortex. However, it is difficult to reconcile Lebensohn's (1936) high values for sodium in parts of the lens with the much lower value previously reported by Fischer (1933) for the whole lens.

In order to resolve this disagreement, we have measured the sodium and potassium concentrations in the capsule (with epithelium), and in concentric layers of the cattle lens, under various conditions. The results show that the concentration of sodium in the nucleus of the lens is actually lower than that in the cortex.

METHODS

Materials. The lenses of cattle (calves or older beasts) were used. Hereafter these will be referred to as calf and cattle lens respectively. Eyes were removed from the animal within a few minutes of death. Usually the lens was removed from the eye and dissected or frozen within an hour of death. The cornea was removed, the iris and ciliary body were stripped off and the zonule was cut through with scissors. The lens was then gently lifted out with the fingers; in this way the lens could be separated completely from the vitreous humour. In early experiments the lens was freed as far as possible from aqueous humour by moving it about and rolling it over on a Petri dish until it no longer left a wet trail, but in later experiments it was rolled on filter paper until it was quite dry.

Dissection of lens. In early experiments the lens was pierced through the centre with one prong of a pair of fine forceps, and a uniform layer of tissue was scraped off with a sharp scalpel and transferred to a weighed tube (10 ml., graduated) with a ground-glass stopper. Sometimes several successive concentric layers were removed in this way, and finally the central core was transferred to a weighed tube. After reweighing, the tubes were stoppered and put aside for analysis of the tissue by flame photometry. In all experiments the whole lens was analysed, either entire or in its separate layers. Very small amounts of the sticky tissue were lost because they adhered to the instruments.

In later experiments the lens was frozen in solid CO_2, having been pierced with a sharp needle which was threaded with coloured cotton for subsequent identification of the lens. Layers of lens were removed as before, but with this technique it was possible to chip off much thinner layers. In some experiments a slit was made in the posterior surface of the capsule, which was then stripped off with fine forceps, before plunging the rest of the lens into solid CO_2. The capsule, removed in this way, takes with it some or all of the epithelial cells under the front surface and also a few of the outermost lens fibres. In some experiments the lens was removed from the eye at the slaughterhouse, blotted and plunged into solid CO_2 within 2 min. of the death of the animal.

A difficulty in these experiments was to remove equivalent layers of tissue from the two lenses of an animal in order to make a valid comparison between them. The degree of success obtained can be judged partly from the weight of tissue in comparable layers, but even with layers of the same weight there must be some error since no layer was uniformly thick all round. The results of the experiments reported in this paper are, however, sufficiently definite for this objection not to matter.

Storage of lens at 4°. One lens of a pair of calf lenses was divided into two parts (outer layer and nucleus) and weighed, and the other was placed in a small dry beaker, standing in a sealed glass jar containing 0·9% NaCl soln. The salt solution, having approximately the same vapour pressure as the fluid of the lens, was added to the jar to minimize the loss or gain of water by the lens during storage. After 24 hr. at 4° this lens was divided into two parts for analysis.

Estimation of the sodium and potassium contents of the lens. The samples of lens tissue were dried at 105° and reweighed to obtain the dry weight and the weight of water lost by drying. About 5 vol. of conc. HNO_3 was added to each tube (0·2–2·0 ml.) and, after lightly stoppering, the tubes were placed upright on an electric hot-plate with a constant-temperature attachment. The temperature was raised during 30 min. to 100°, maintained for 3 hr. and finally raised to 130° for 1 hr. After cooling, the digests were diluted with water (usually to 10 ml.) and if cloudy were clarified by centrifuging. Sodium and potassium were determined in the suitably diluted digests by means of a lithium internal-standard flame photometer (Amoore, Parsons & Werkheiser, 1958). The digests were usually diluted so that the final concn. of HNO_3 was not above 0·3N, thereby avoiding any interference by the acid with the estimation of sodium and potassium. In the presence of 0·6N-HNO_3 (which was present in the analyses of lens capsules) a 5% depression of the readings for potassium was observed. A correction was made in these cases by adding the same concentration of HNO_3 to the standard potassium solutions used for calibrating the flame photometer.

Expression of results. Throughout this paper the amounts of sodium and potassium in the lens are expressed in relation to the amounts of water in the lens as millimolal concentrations. Frequently in the literature the cation contents of a tissue are expressed as a quantity in a defined weight of fresh tissue. This method of expression is of limited value unless the water content of the tissue is also given, since tissues vary greatly in their degree of hydration under different experimental conditions. The amount of a cation may also be expressed in relation to the amount of dry matter of the tissue. This is often useful when ion-binding is studied. Hence the amount of water related to a fixed amount of tissue (l. of water/kg. dry wt.) is also given in all tables to enable comparisons of ion content and dry matter to be made.

RESULTS

Sodium, potassium and water contents of concentric layers of calf lens. The analyses of four calf lenses, divided into concentric layers, are shown in Table 1. The water content of the lens nucleus was substantially less than that of the cortex (see also Ullberg-Olsson, 1951). The concentration of sodium showed a progressive decrease from the periphery towards the centre of the lens. When the lens was divided into cortex and nucleus of approximately equal weights, the concentration of sodium in the former was usually about 20 m-molal, and the concentration in the latter about 12 m-molal (first three lenses, Table 1). A higher value of 32 m-molal sodium was obtained when a thin outermost layer of the cortex was analysed (fourth lens, Table 1). The concentration of potassium in the lens ranged between 111 and 126 m-molal. Although there were no pronounced differences between the concentrations of potassium in successive layers, this cation was frequently more concentrated in the intermediate layers of the lens than it was in the outermost layer or in the nucleus (fourth lens, Table 1). The overall concentration of sodium and of potassium in the whole lens ranged from 15 to 19 m-molal and from 119 to 122 m-molal respectively. The analyses of calf lens by Fischer (1933) and by Mandel, Nordmann & Bloch (1953) are in rough agreement with the present work for potassium content, but these workers reported about twice as high a sodium content as we have observed.

Sodium, potassium and water contents of cattle lens. Table 2 gives the corresponding data for the lenses of cattle. The water content of the adult lens was somewhat less than that of the calf lens (1·8 l. of water/kg. dry wt. compared with 2·1 l. of water/kg. dry wt.). However, the distribution of sodium and potassium within the lens was not

markedly different. The concentration of sodium in the cortex of the adult lens was somewhat higher than that in the calf lens (30 compared with 20 m-molal), and the potassium concentration was slightly lower (114 compared with 120 m-molal). Overall the adult lens had the same concentration of potassium as the calf lens, but the concentration of sodium was somewhat higher (21 compared with about 17 m-molal). All our values for the concentration of sodium within the cattle lens are in direct contrast to those of Lebensohn (1936), both for absolute concentrations and the direction of the concentration gradient within the lens.

Effect of blotting on the sodium and potassium contents of calf lens. As the lens is in contact with the aqueous humour, it was thought possible that the presence of higher concentrations of sodium in the cortex might be due to traces of this fluid adhering to the lens. Our measurements of the cation content of cattle aqueous humour gave Na^+ ions 146 m-molal and K^+ ions 4·4 m-molal, and of vitreous humour gave Na^+ ions 142 m-molal and K^+ ions 5·5 m-molal, in good agreement with Tron (1926, 1927). To account for the observed difference between the concentrations of sodium in cortex and nucleus would require that 13–23 μl. of aqueous humour was left adhering to the lens after it had been dried as far as possible by rolling it on a Petri dish. This seems very unlikely, because even 1 μl. of liquid on a glass plate is easily seen when spread over an area of 8 cm.^{2} However, further analyses were made of the calf lens after it had been thoroughly blotted by rolling on filter paper as previously described. The results in Table 3 show that the sodium concentration in the outermost layer of the lens was slightly reduced by this treatment, compared with the unblotted lens in Table 1. The reduction in sodium concentration might have been due to the removal of the last traces of aqueous humour from the surface of the lens, or possibly to the withdrawal of some fluid from within the lens. The blotting procedure was subsequently adopted as routine because it was

Table 1. *Sodium and potassium contents of concentric layers of calf lens*

The lens was removed from the eye within 1 hr. of death of the calf, freed from aqueous humour as far as possible without blotting and dissected at room temperature.

Portion of lens analysed	Wet wt. (g.)	Water content (l./kg. dry wt.)		Concn. of cation (m-molal)			
				Sodium		Potassium	
		Zone	Whole lens	Zone	Whole lens	Zone	Whole lens
Outer zone, i.c.*	0·4986	2·68	2·16	20·8	18·9	118	119
Inner zone	0·4914	1·62		15·2		121	
Outer zone, i.c.	0·4914	2·70	2·12	21·5	18·3	121	122
Inner zone	0·3153	1·51		12·2		126	
Outer zone, i.c.	0·4920	2·84	2·18	18·3	15·9	122	121
Inner zone	0·3047	1·43		11·0		119	
Outermost zone, i.c.	0·1316	3·67	2·02	32·0	15·1	116	119
Second layer	0·1524	2·86		14·0		123	
Third layer	0·1797	2·32		9·8		126	
Fourth layer	0·1724	1·79		11·3		120	
Fifth layer	0·1323	1·51		11·0		116	
Innermost zone	0·1286	1·28		11·8		111	

* In this and other tables 'i.c.' means 'including capsule and adhering tissue'.

Table 2. *Sodium and potassium contents of concentric layers of cattle lens*

Details are as given in Table 1, except that the lenses were from adult cattle.

Age of animal (years)	Portion of lens analysed	Wet wt. (g.)	Water content (l./kg. dry wt.)		Concn. of cation (m-molal)			
					Sodium		Potassium	
			Zone	Whole lens	Zone	Whole lens	Zone	Whole lens
4	Outer zone, i.c.	0·9239	3·12	1·87	28·5	21·8	114	120
	Second layer	0·9720	1·65		17·6		127	
	Innermost zone	0·4846	1·07		13·5		121	
9	Outer zone, i.c.	0·8377	3·11	1·77	31·1	20·4	114	122
	Second layer	0·9738	1·67		13·8		125	
	Innermost zone	0·7012	1·08		12·5		129	

Table 3. *Effect of blotting calf lens before analysis*

Details are as given in Table 1, except that the lens was blotted with filter paper until every trace of moisture had been removed.

Portion of lens analysed	Wet wt. (g.)	Water content (l./kg. dry wt.)	Concn. of cation (m-molal)	
			Sodium	Potassium
Outer zone, i.c.	0·4187	3·13	15·3	125
Inner zone	0·5396	1·70	10·7	129
Outer zone, i.c.	0·4770	3·12	13·4	126
Inner zone	0·5371	1·68	9·1	134
Outermost zone, i.c.	0·0929	3·65	22·6	117
Second layer	0·1023	2·84	15·2	125
Third layer	0·1776	2·36	14·0	122
Innermost zone	0·4947	1·65	12·8	122
Outermost zone, i.c.	0·0935	2·94	17·5	136
Second layer	0·0749	2·86	13·5	126
Third layer	0·1033	2·58	15·5	127
Fourth layer	0·2368	2·28	12·6	123
Fifth layer	0·1578	1·61	9·0	120
Innermost zone	0·1450	1·26	8·3	119

Table 4. *Effect of washing calf lens in water or* 0·25 M-*sucrose*

The lenses were washed for 1 min. in water or 0·25 M-sucrose before blotting and dissection.

Washing medium	Portion of lens analysed	Wet wt. (g.)	Water content (l./kg. dry wt.)	Concn. of cation (m-molal)	
				Sodium	Potassium
Water	Outer zone, i.c.	0·5171	2·98	11·8	116
	Inner zone	0·4094	1·49	11·1	117
Water	Outer zone, i.c.	0·5925	2·90	11·8	112
	Inner zone	0·3868	1·48	10·4	104
Water	Outer zone, i.c.	0·6129	2·82	13·4	124
	Inner zone	0·4245	1·48	11·4	112
Water	Outer zone, i.c.	0·4859	2·77	12·7	114
	Inner zone	0·3792	1·48	12·8	110
Water	Outer zone, i.c.	0·2022	3·92	11·5	106
	Second layer	0·2266	2·68	12·4	118
	Third layer	0·1382	2·30	12·7	122
	Inner zone	0·3872	1·51	15·5	130
0·25 M-Sucrose	Outer zone, i.c.	0·1681	3·25	9·0	130
	Second layer	0·1205	2·82	9·5	121
	Third layer	0·1886	2·40	13·1	122
	Inner zone	0·4074	1·54	14·8	122

desirable that possible superficial contamination by aqueous humour should be minimized. The excess of sodium content, which withstands blotting, must lie below the surface of the lens.

Effect of washing calf lens before analysis. Table 4 shows that rinsing the lens for 1 min. in water or in 0·25 M-sucrose soln. reduced the concentration of sodium in the outside layer to a value nearly the same as, or even lower than, that in the centre of the lens. Thus the excess of sodium beneath the surface of the lens is easily washed out.

Analysis of the lens capsule plus adhering cells and fibres. Table 5 shows that the lens capsule, plus adhering cells and fibres, had a very much higher sodium concentration than the rest of the lens (82–98 m-molal compared with about 14 m-molal). Therefore the excess of sodium in the outer layer of the lens is mainly localized in the capsule and adhering tissue. Nevertheless, this high concentration of sodium cannot be entirely responsible for the observed progressive decrease in sodium concentration towards the centre of the lens (Tables 1–3), because a (diminished) concentration gradient persisted within the decapsulated lens (Table 6).

Effect of storing the lens at 4°. During storage of the blotted excised lens in a moist atmosphere for 24 hr. at 4° there was a slight loss of water from the lens (Table 7). There was also a marked reduction in the concentration gradient of sodium. Evidently some of the excess of sodium in the outer zone must

have diffused towards the centre of the lens. The decrease in the sodium-concentration gradient under these conditions shows that the gradient demonstrated in fresh lens cannot be supported by structural factors alone (such as impermeable membranes or binding of ions), but must be maintained *in vivo* by some active process which counteracts diffusion.

Post-mortem changes. As the lens depends upon the relatively slowly flowing aqueous humour for its nutrients, it might be expected that post-mortem changes would be less immediate than in organs more directly dependent upon blood supply. One lens of each pair of eyes was excised, blotted and plunged into liquid air within 2 min. of the death of the calf. The other eye was kept at the

Table 5. *Sodium and potassium in calf-lens capsule*

A pair of lenses from one animal were thoroughly blotted. One lens was analysed whole; the other was decapsulated and the capsule plus adhering tissue was analysed separately from the rest of the lens.

Sample analysed	Wet wt. (g.)	Water content (l./kg. dry wt.)	Concn. of cation (m-molal)	
			Sodium	Potassium
Whole lens	0·9863	2·08	14·6	126
Capsule	0·0126	4·25	98	98
The rest	0·9610	2·00	13·5	130
Whole lens	0·8934	2·04	14·7	129
Capsule	0·0120	4·70	82	88
The rest	0·8970	2·02	13·1	127
Whole lens	0·9647	2·02	15·3	127
Capsule	0·0126	4·04	93	101
The rest	0·9445	1·94	14·1	130

Table 6. *Sodium and potassium in the capsule and in concentric layers of calf lens*

The lens was thoroughly blotted, decapsulated and frozen in solid CO_2. Two concentric layers and the remaining nucleus were analysed.

Portion of lens analysed	Wet wt. (g.)	Water content (l./kg. dry wt.)	Concn. of cation (m-molal)		
			Sodium	Potassium	Sodium + potassium
Capsule	0·0179	4·57	54·3	114	168·3
Outer layer	0·1134	3·00	16·1	134	150·1
Second layer	0·2326	2·44	12·5	133	145·5
Nucleus	0·4383	1·69	10·5	122	132·5
Capsule	0·0185	4·62	72·4	104	176·4
Outer layer	0·1244	3·14	16·4	142	158·4
Second layer	0·1812	2·38	13·8	133	146·8
Nucleus	0·4829	1·69	10·6	126	136·6
Capsule	0·0125	4·67	78·9	101	179·9
Outer layer	0·0847	2·91	15·8	140	155·8
Second layer	0·2842	2·79	13·1	130	143·1
Nucleus	0·5137	1·70	11·3	124	135·3

Table 7. *Effect of storage at 4° on isolated calf lens*

One lens of each pair was dissected for analysis within an hour of death; the other was removed from the eye and refrigerated for 24 hr. at 4° before being dissected. Both lenses were thoroughly blotted.

Time of storage at 4° (hr.)	Portion of lens analysed	Wet wt. (g.)	Water content (l./kg. dry wt.)	Concn. of cation (m-molal)	
				Sodium	Potassium
0	Outer zone, i.c.	0·4187	3·13	15·3	125
	Inner zone	0·5396	1·70	10·7	129
24	Outer zone, i.c.	0·5439	2·76	13·4	131
	Inner zone	0·3959	1·59	11·5	137
0	Outer zone, i.c.	0·4770	3·11	13·4	126
	Inner zone	0·5371	1·68	9·1	134
24	Outer zone, i.c.	0·5140	2·96	12·1	135
	Inner zone	0·4920	1·56	11·5	130

Table 8. *Comparison of sodium and potassium contents of calf lens frozen within 2 min. of death and lens kept in the eye for some hours before freezing*

One lens of a pair was removed from the eye, blotted and frozen within 2 min. of death. The other eye was kept at about 18° for the stated length of time before the lens was removed, blotted and frozen. Two thin concentric layers and the remaining nucleus were analysed.

Interval between death of animal and removal of lens	Portion of lens analysed	Wet wt. (g.)	Water content (l./kg. dry wt.)	Concn. of cation (m-molal)	
				Sodium	Potassium
2 min.	Outermost layer, i.c.	0·0810	3·70	24·2	144
	Second layer	0·2273	2·67	16·9	132
	Remaining nucleus	0·6061	1·75	11·3	130
1 hr.	Outermost layer, i.c.	0·0754	3·42	12·8	137
	Second layer	0·2054	2·16	9·6	130
	Remaining nucleus	0·5701	1·71	10·7	120
2 min.	Outermost layer, i.c.	0·0930	3·42	18·8	139
	Second layer	0·2070	3·04	9·7	131
	Remaining nucleus	0·5573	1·77	8·0	131
3 hr.	Outermost layer, i.c.	0·0622	4·18	12·0	134
	Second layer	0·1970	2·95	10·8	129
	Remaining nucleus	0·6008	1·67	10·6	123
2 min.	Outermost layer, i.c.	0·1143	3·54	15·2	146
	Second layer	0·1806	2·79	8·3	131
	Remaining nucleus	0·5792	1·52	9·9	126
5 hr.	Outermost layer, i.c.	0·1558	3·58	35·4	111
	Second layer	0·1390	2·88	29·2	111
	Remaining nucleus	0·5858	1·75	18·2	110
18 hr.	Outermost layer, i.c.	0·1118	3·66	72	97
	Second layer	0·2553	2·57	76	98
	Remaining nucleus	0·4167	1·52	62	92

Table 9. *Sodium and potassium contents of concentric layers of adult rabbit lens*

Details are as given in Table 1, except that the lenses were from old rabbits. The corresponding portions of the pairs of lenses were combined for analysis.

Age of animal (months)	Portion of lenses analysed	Wet wt. (g.)	Water content (l./kg. dry wt.)		Concn. of cation (m-molal)			
					Sodium		Potassium	
			Zone	Whole lens	Zone	Whole lens	Zone	Whole lens
31	Outer zones, i.c.	0·6310	2·64		39·5		111	
	Second layers	0·3728	1·19	1·59	15·1	28·9	119	112
	Innermost zones	0·2706	0·83		12·1		101	
40	Outer zones, i.c.	0·5450	2·75		33·7		114	
	Second layers	0·3800	1·35	1·51	16·3	24·0	126	114
	Innermost zones	0·4095	0·83		13·1		103	

ambient temperature for a known length of time before the lens was removed, blotted and frozen. It was not possible to strip off the capsule from the frozen lens, but as thin an outermost layer as possible was removed, followed by a second thin layer. Table 8 shows that after 5 and 18 hr. at 18° the sodium content increased, and the potassium content decreased, throughout the lens. The delay of at least 3 hr. before sodium began to pervade the lens suggests that this ion was initially excluded from the lens by an active process which subsequently failed.

Potassium content of the lens. The potassium concentration was lowest in the capsule plus adhering tissue, where the sodium concentration was highest (Tables 5 and 6). It was highest in the layers just beneath the capsule, whereas in the rest of the lens the concentration of potassium, like that of sodium, decreased towards the centre (Table 6). The distribution of potassium within the lens was obscured in the other tables because the regions of lowest concentration (the capsule plus adhering tissue) and highest concentration (the fibres just below the capsule) were both included in the 'outer layer'.

The sodium and potassium concentrations both decreased towards the centre of the lens, so that the cation concentration was some 20 m-molal lower in the centre of the lens than it was in the peripheral fibres immediately below the capsule, and about 40 m-molal below that in the capsule itself (Table 6, last column).

Sodium, potassium and water contents of adult rabbit lens. The analyses of the lenses from two old rabbits are given in Table 9. The rabbit lens showed qualitatively the same features as those from calves and cattle (Tables 1 and 2), but the concentration gradient of sodium was even more pronounced. The overall sodium and potassium concentrations in old rabbit lens agree with those reported by Heinrichs & Harris (1957) for younger rabbits.

DISCUSSION

Our values for the total sodium contents of cattle or calf lenses are not in good agreement with Fischer (1933) or Mandel *et al.* (1953). As our values are lower in both cases, it may be that the results of these workers were influenced by contamination with aqueous humour or by post-mortem changes. Langham & Davson (1949) noted that the chloride content of the rabbit lens, left in the eye, began to increase 4 hr. after death. These changes were qualitatively similar to the post-mortem penetration of sodium into calf lens shown in Table 8.

The data of Lebensohn (1936) remain completely at variance with those of Fischer (1933), Mandel *et al.* (1953) and the present work (Tables 1 and 2) for the total sodium content of cattle lens. Furthermore, the present work (Tables 1–3 and 6) always demonstrates that the direction of the concentration gradient of sodium within the lens is actually opposite to that claimed by Lebensohn. If Lebensohn's (1936) data are incorrect, Langham & Davson's (1949) conclusions about the distribution of chloride in lens may need revising, because they used Lebensohn's data on sodium distribution in order to interpret their own analyses of total chloride content.

The effects of blotting or briefly rinsing the lens before analysis (Tables 3 and 4) suggest that just below the surface of the isolated lens is an accessible space, containing sodium-rich fluid, and in ready communication with the exterior. The volume of this space is about 1 % of the total volume of the lens. This sodium-rich space is largely associated with the capsule and adherent cells (Tables 5 and 6). Analysis of whole, blotted calf lens (Table 5) shows that it contains only about 10 % of the concentration of sodium in the aqueous humour. Assuming that this sodium is extracellular, then the extracellular space of the calf lens is 6–7 % of its total volume. This value agrees well with the extracellular spaces of sheep and rabbit lens measured by Langham & Davson (1949) with chloride, ^{24}Na, ^{82}Br and sucrose. The decrease in sodium concentration towards the centre of the lens (Tables 1–3 and 6) may be due to the closer packing of the fibres in the nucleus of the lens, with a consequent reduction in extracellular space. However, it is quite possible that some of the sodium in the lens is actually intracellular.

Our experiments have demonstrated that diffusion of sodium can take place within the lens (Table 7), and that sodium can diffuse into the lens from the aqueous humour, while potassium escapes from the lens (Table 8). Both these experiments were conducted under conditions expected to interfere with metabolism (cooling to 4°, or prolonged storage at 18° in the eye). However, experiments carried out *in vivo* with systemic injection of radioactive tracers have shown that the rabbit lens is slightly permeable to ^{24}Na (Langham & Davson, 1949; von Sallmann & Locke, 1951) and to ^{42}K (von Sallmann & Locke, 1951). Accordingly, the observed differences in sodium and potassium concentration between the lens and the aqueous humour, and the concentration gradients within the lens, must be actively maintained *in vivo*.

Our analyses show that there is a continuously decreasing concentration of sodium from the surface to the centre of the lens. This could arise from a passive leak of sodium from the aqueous humour into the lens, opposed by an active radial transport of sodium outwards. This is in accordance with the work of Harris & Gehrsitz (1951), who showed that the rabbit lens, incubated at 5–7° in a medium of ionic content similar to that of aqueous humour, loses K^+ and gains Na^+ ions. Reversal of this process takes place if the lens is subsequently incubated at 37° (Harris, Gehrsitz & Nordquist, 1953); this reversal is prevented by lack of glucose or oxygen and by other treatments expected to interfere with normal lens metabolism (Harris, Hauschildt & Nordquist, 1954*a*, *b*). According to Heinrichs & Harris (1957) the active transport of sodium and potassium in opposite directions may not necessarily be coupled. Our experiments showing a continuous concentration gradient suggest that it is not only at the lens capsule that sodium is transported outwards, but also that each successive layer of fibres has some power to transport sodium from the nucleus towards the cortex.

SUMMARY

1. The sodium, potassium and water contents of the capsule plus adhering tissue, and of concentric layers of calf and cattle lens, were measured under various conditions.

2. The average concentration of sodium in calf or cattle lens was 15–21 m-molal, and of potassium 119–122 m-molal.

3. The sodium concentration in calf or cattle lens increased from about 12 m-molal in the nucleus to about 80 m-molal in the capsule plus adhering tissue. The potassium concentration was maximal in the cortical fibres just below the capsule.

4. The excess of concentration of sodium in the outermost zone was reduced by blotting the lens, and was abolished by washing the lens in water or 0·25 M-sucrose.

5. Storage of the excised lens at 4° caused the sodium to become more evenly distributed throughout the tissue water.

6. Storage of the lens within the excised eye at 18° for periods up to 18 hr. caused an inward penetration of sodium from the aqueous humour, and a loss of potassium from the lens. The penetration started after storage for about 3 hr.

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Studies on Sulphatases

24. THE USE OF BARIUM CHLORANILATE IN THE DETERMINATION OF ENZYMICALLY LIBERATED SULPHATE*

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The benzidine micro-method (Dodgson & Spencer, 1953) has been used extensively in these Laboratories for the determination of enzymically liberated sulphate. However, as indicated by Dodgson & Spencer (1957), the procedure is both exacting and time-consuming. Consequently, the need has arisen for the development of a simpler method which can more readily be applied to the routine determination of a large number of samples. Such a method, based on the use of barium chloranilate (see Bertolacini & Barney, 1957), has now been devised.

EXPERIMENTAL AND RESULTS

Materials

Barium chloranilate. This reagent was prepared from chloranilic acid (2:5-dichloro-3:6-dihydroxy-*p*-benzoquinone; L. Light and Co.) and barium chloride, by the procedure of Bertolacini & Barney (1957). Commercial preparations of the reagent were also used, but these tended to give high blank values. These values could be minimized by washing the barium chloranilate several times by suspension in water, followed by filtering, washing with acetone and drying *in vacuo* at 50°. All preparations of barium chloranilate were finely powdered before use.

Tissue and enzyme preparations. Tissue suspensions were prepared according to Dodgson, Spencer & Thomas (1953). *Proteus vulgaris* chondrosulphatase was prepared by the method of Dodgson & Lloyd (1957), and glycosulphatase was obtained from extracts of the digestive gland of *Littorina littorea* by chromatography on diethylaminoethylcellulose (A. G. Lloyd, unpublished work).

Carbohydrate sulphates. Potassium glucose 3-sulphate was prepared by a modification of the method of Duff (1949); the potassium salts of glucose 6-sulphate, *N*-acetylglucosamine 6-sulphate and *N*-acetylgalactosamine monosulphate were prepared by the method of Lloyd (1959). Potassium chondroitin sulphate was obtained from ox tracheal cartilage by the method of Dodgson, Lloyd & Spencer (1957); heparin was a commercial preparation (British Drug Houses Ltd.).

* Part 23: Dodgson, Rose & Tudball (1958).
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All stages of the procedure were performed in 3 ml. Pyrex test tubes chosen for uniformity of bore. These tubes were suitable for the centrifuging steps when used in conjunction with an MSE 32-place angle-head centrifuge (catalogue no. 6885; Measuring and Scientific Equipment Ltd.). All glassware was cleaned with nitric acid; the use of sulphuric acid-containing mixtures was avoided. Reaction mixtures were agitated by holding the tube against a rapidly rotating flattened rod. A $\frac{1}{12}$ h.p. electric motor, with a flattened spindle, operating at 500–1000 rev./min., was suitable for this purpose.

Methods

Determination of sulphate in aqueous solution. In a 3 ml. test tube, 0·2 ml. of an aqueous solution of K_2SO_4 (5–80 μg. of SO_4^{2-} ions/0·2 ml.) was mixed with 0·8 ml. of ethanol. A portion (0·8 ml.) of the ethanolic sulphate solution was withdrawn and mixed with 0·2 ml. of aqueous 0·05 M-potassium hydrogen phthalate solution, pH 4·0. To this was added 0·5 ml. of a suspension of barium chloranilate (approx. 400 μg./0·5 ml.) in 0·25 M-sodium acetate–acetic acid solution, pH 4·0. Since the barium chloranilate is only slightly soluble and is always present in excess, the addition of an exact weight is unnecessary. The tube was capped with Parafilm (V. A. Howe and Co.) and the mixture was briefly agitated at 1 min. intervals for a total of 20 min. Excess of barium chloranilate and precipitated barium sulphate were removed by centrifuging at 4000 *g* and 0° for 10 min. The amount of chloranilate ion released into solution was measured against a reagent blank in the Uvispek spectrophotometer at 350 mμ in 1 cm. cells. The cell carrier of the instrument was modified to permit the use of 1 cm. cells with the 1·5 ml. test volume obtained in the above procedure. A straight-line relationship was obtained between the concentration of sulphate and the spectrophotometric reading.

Quantitative recoveries of sulphate were obtained when the sulphate standards were prepared in the presence of sodium acetate–acetic acid solution up to a concentration of 0·25 M at pH values within the range 3·0–7·5. To estimate sulphate in the presence of buffered tissue and enzyme preparations, a portion (0·1 ml.) of a solution of K_2SO_4 (20–30 μg. of SO_4^{2-} ions/0·1 ml.) in 0·2 M-sodium acetate–acetic acid solution, pH 5·5, was mixed with 0·1 ml. of a tissue or enzyme preparation in the same medium. A suitably diluted tissue or enzyme preparation was used as a control. After incubation for 1 hr. at 37·5°, 0·8 ml. of ethanol was added and, after mixing, the whole was allowed to stand at 0° for 10 min. Precipitated protein, and polysaccharide where present, was removed by centrifuging at 3500 *g* and 0° for 15 min. A portion (0·8 ml.) of the clear supernatant was withdrawn and the inorganic sulphate determined as above. Sulphate could be recovered quantitatively from all the tissue and enzyme preparations tested (see Table 1). Na^+, K^+, NH_4^+, Mg^{2+}, Cl^-, F^-, CN^-, PO_4^{3-}, HCO_3^- and oxalate ions when present in concentrations up to 0·02 M did not interfere with the recovery of sulphate from acetate-buffered tissue suspensions. However, Cu^{2+}, Co^{3+}, Mn^{2+}, Zn^{2+}, Fe^{3+}, Pb^{2+} and Ca^{2+} ions at the same concentration all interfered strongly (Table 2). The presence of the potassium salts of glucose 6-sulphate (0·02 M), glucose 3-sulphate (0·02 M), *N*-acetylglucosamine 6-sulphate (0·015 M), *N*-acetylgalactosamine monosulphate (0·015 M), chondroitin sulphate (0·25 %, w/v) and heparin (0·23 %, w/v) did not affect the recoveries of sulphate. However, increase of the concentration of both chondroitin sulphate and heparin above the values quoted decreased the recovery of added inorganic sulphate.

Assay of glyco- and chondro-sulphatase. As a check of the method the enzymic release of sulphate from potassium glucose 6-sulphate and potassium chondroitin sulphate by glyco- and chondro-sulphatase respectively was examined. Experimental details are given in Table 3. The results obtained by the chloranilate procedure were compared with a similar series of determinations made simultaneously with the benzidine micro-method (Dodgson & Spencer, 1953). In each experiment four sets of six test determinations and six control determinations were made. After incubating each set for the time interval indicated and precipitating protein and polysaccharide by the addition of ethanol, the inorganic sulphate in three tests and three controls was determined by the chloranilate procedure, and inorganic sulphate in the remaining tests and controls was determined by the benzidine method. Table 3 shows that good agreement was obtained between the two methods.

Table 1. *Recovery of potassium sulphate from tissue and enzyme preparations after incubation for 1 hr. at 37°*

Preparation	Final concn. of suspension (g. dry wt./100 ml.)	SO_4^{2-} ions added (μg.)	Recovery (%)
Rat			
Liver	1·8	25·8	98·2
Kidney	2·1	25·8	100·8
Spleen	2·0	25·8	99·4
Spleen	1·9	30·4	104·1
Lung	2·1	25·8	98·6
Cartilage	2·0	30·4	99·8
Pancreas	1·8	30·4	97·6
Human			
Liver	2·0	22·6	99·1
Kidney	2·0	22·6	99·6
Proteus vulgaris			
Chondrosulphatase	0·1	22·6	99·6
Littorina littorea			
Glycosulphatase	0·5	22·6	101·5

Table 2. *Recovery of potassium sulphate in the presence of inorganic ions*

Compound added	Concn. (M)	SO_4^{2-} ions added (μg.)	Recovery (%)
NaCl	0·02	22·0	98·0
KCl	0·02	22·0	97·2
$MgCl_2$	0·02	22·0	96·8
NH_4Cl	0·02	35·8	14·5
$CaCl_2$	0·02	35·8	9·8
$CoCl_2$	0·019	35·8	11·5
$MnCl_2$	0·02	35·8	0
$FeCl_3$	0·02	35·8	0
$PbCl_2$	0·02	35·8	7·4
$CuCl_2$	0·18	35·8	8·2
NaF	0·02	30·4	101·4
NaH_2PO_4	0·02	30·4	98·6
$NaHCO_3$	0·02	30·4	104·8
Sodium oxalate	0·02	30·4	101·9

Table 3. *Comparison of the benzidine and barium chloranilate methods for the determination of* SO_4^{2-} *ions liberated by carbohydrate sulphatases*

(*a*) A portion (0·1 ml.) of potassium chondroitin sulphate solution (0·25%, w/v, in 0·2M-sodium acetate–acetic acid solution, pH 7·0) was incubated at 37·5° with 0·1 ml. of *Proteus vulgaris* chondrosulphatase (0·1%, w/v, in 0·2M-sodium acetate–acetic acid solution, pH 7·0). At the end of the incubation period 0·8 ml. of ethanol was added and inorganic sulphate released by enzyme action was determined. Suitable control determinations were made in which enzyme and substrate were incubated separately and mixed only immediately before the addition of ethanol.

Incubation period (min.)	SO_4^{2-} ions liberated (μg.)	
	Chloranilate method	Benzidine method
10	15·6	16·4
20	28·8	27·4
30	40·4	40·8
40	48·8	51·2

(*b*) Potassium glucose 6-sulphate (0·1 ml. of 0·01M solution in acetate buffer, pH 5·5) was incubated at 37·5° with 0·1 ml. of *Littorina littorea* glycosulphatase (0·5%, w/v, in the same buffer). Control determinations were made as described above.

Incubation period (min.)	SO_4^{2-} ions liberated (μg.)	
	Chloranilate method	Benzidine method
10	8·4	9·8
20	17·6	18·2
30	26·3	25·8
40	35·7	35·3

DISCUSSION

The chloranilate procedure for the determination of sulphate compares favourably with the benzidine method for the assay of both glyco- and chondrosulphatase. The validity of the benzidine method as a means of estimating enzymically released sulphate has already been amply demonstrated (Dodgson & Spencer, 1953). The principal advantage of the present method is the considerable reduction in the time required to complete the assay of such carbohydrate sulphatases. The procedure is particularly useful therefore for the determination of a large number of samples as required, for example, in chromatographic studies on these sulphatases. However, at the present stage of development it offers no increase in sensitivity as compared with the benzidine method.

The introduction of the ethanol-precipitation step serves a double purpose. First, treatment of incubation mixtures with ethanol to 80% (v/v) results in the precipitation of both protein and polysaccharide which might otherwise interfere with subsequent stages in the procedure. Secondly, as originally noted by Bertolacini & Barney (1957), the presence of ethanol in the test solution results in a decrease in the solubility of both barium sulphate and barium chloranilate. In aqueous solution the solubilities of barium sulphate and barium chloranilate are $9·6 \times 10^{-6}$ and $2·2 \times 10^{-4}$ mole/l. respectively. In aqueous 50% (v/v) ethanol these values are reduced to $2·5 \times 10^{-7}$ and $5·2 \times 10^{-6}$ mole/l. respectively.

The determination of sulphate by this method is not adversely affected by a number of common anions used in enzymology. However, it is unfortunate that, with the exception of certain univalent ions, cation interference is rather pronounced.

The variations in experimental conditions described here are not exhaustive, and further information about the performance of the technique will become available as the use of the method is extended. In general it has been found advisable to test the method (by recovery of added potassium sulphate) whenever new conditions are employed. The effect of buffer solutions other than acetate has not been examined here since the majority of workers on sulphatases have preferred this type of incubation medium. For similar reasons, no attempt has been made to examine the effect of values higher than pH 7·5 or lower than pH 3·0 for the incubation mixtures, since the range pH 3·0–7·5 embraces the pH optima of all the carbohydrate sulphatases at present known.

SUMMARY

1. A modification of the chloranilate procedure for determining small amounts (5–80 μg./0·2 ml.) of enzymically liberated inorganic sulphate is described.

2. With the method potassium sulphate has been recovered quantitatively from a number of different enzyme and tissue preparations.

3. Recoveries of sulphate are unaffected by the presence of a number of common anions. On the other hand, interference by cations is rather pronounced. Within certain limits recoveries of sulphate are unaffected by the presence of the assay substrates for carbohydrate sulphatases.

4. The procedure has been compared with the benzidine method in the determination of the release of sulphate from glucose 6-sulphate and chondroitin sulphate by glycosulphatase and chondrosulphatase respectively.

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Location of ^{14}C in Protein from Isolated Rat Diaphragm Incubated *in vitro* with [^{14}C]Amino Acids and with $^{14}CO_2$

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When isolated rat diaphragm is incubated *in vitro* with radioactively labelled amino acids, radioactive material is transferred to the protein (the trichloroacetic acid-insoluble portion) of the diaphragm (Sinex, MacMullen & Hastings, 1952; Krahl, 1953; Manchester & Young, 1958*a*). Manchester & Young (1958*b*) found that when rat diaphragm is incubated with [^{14}C]glucose, [^{14}C]-pyruvic acid, [^{14}C]oxoglutaric acid or with $^{14}CO_2$, ^{14}C is incorporated into protein. By the use of column chromatography we have now determined in which amino acids this incorporated ^{14}C is contained, and to what extent [^{14}C]amino acids are converted by diaphragm into other [^{14}C]amino acids before incorporation into protein.

MATERIALS AND METHODS

Radioactive materials. Labelled substances were obtained from The Radiochemical Centre, Amersham, Bucks. Glycine and sodium pyruvate were [1-^{14}C]labelled; L-alanine, L-serine, L-glutamic acid, L-aspartic acid and D-glucose were all uniformly ^{14}C-labelled ([U-^{14}C]). Sodium α-oxoglutarate contained ^{14}C in the C-5 position. [^{14}C]-Glucose had a specific activity of about 67 μc/mg., [^{14}C]-sodium pyruvate about 43 μc/mg., [^{14}C]alanine 36 μc/mg., [^{14}C]glycine 37 μc/mg. and [^{14}C]serine 15 μc/mg. Each of these materials was used at a final concentration of 2·5 μc/ml. [^{14}C]Glutamic acid (specific activity 62 μc/mg.) and [^{14}C]aspartic acid (specific activity 32 μc/mg.) were used at a concentration of 10 μc/ml. The protein from diaphragm incubated with [^{14}C]glutamic acid and with [^{14}C]oxoglutaric acid had been prepared in a previous experiment (Manchester & Young, 1958*b*).

The bicarbonate medium of Gey & Gey (1936), pH 7·4, gassed with $O_2 + CO_2$ (95:5, v/v), was used throughout. The incubation flasks each contained 2 ml. of medium. No unlabelled glucose was added to the medium in any experiment. In the experiment with $^{14}CO_2$, 16 μc of $NaH^{14}CO_3$ was added to each flask, its final specific activity being 160 μc/m-mole.

Preparation of diaphragm. Diaphragm was removed from female rats, of about 120 g. weight, which had starved for 20–24 hr. The two halves of the diaphragm were washed in freshly gassed buffer, then gently blotted and transferred to small conical flasks containing the incubation medium. Two hemidiaphragms were added to each flask. The flasks were gassed, sealed and incubated at 37° in a Dubnoff shaker.

Preparation of protein from diaphragm. After incubation for 2 hr. the diaphragm was removed, blotted and homogenized in a Potter–Elvehjem homogenizer in 10 ml. of 10% trichloroacetic acid. The precipitate was spun down, resuspended in 10 ml. of 10% trichloroacetic acid and heated for 15 min. at 90°. The insoluble material was extracted twice with 1·5 ml. of 0·4N-NaOH, and the insoluble residue rejected (protein content about 5%). The dissolved protein was reprecipitated by the addition of 5 ml. of 10% trichloroacetic acid. It was washed with 2 ml. of ethanol–ether (50:50, v/v), followed by 2 ml. of ether, and was dried in a vacuum desiccator.

Hydrolysis of protein. Protein (10 mg.) in 0·3 ml. of 6N-HCl was sealed in a thick-walled glass tube and heated to 105° for 20–24 hr. The hydrolysate was left to dry in a vacuum desiccator over $CaCl_2$ and redissolved in 1 ml. of 1·5N-HCl.

Chromatographic separation of amino acids. The amino acids in the protein hydrolysate were separated on a column (50 cm. × 1 cm.) of either Dowex-50 or Amberlite CG-120. The resin of both columns had been prepared for use by the method of Moore & Stein (1951), and was prepared for each experiment by washing with 2N-NaOH, followed by water and 1·5N-HCl. Washing with HCl was continued until the effluent left no deposit of NaCl when evaporated to dryness. The protein hydrolysate was introduced at the top of the column and solvent run through at room temperature at the rate of about 4 ml./hr. As solvents 1·5N-, 2·5N- and 4N-HCl were successively used (Stein & Moore, 1949). The eluate was collected in fractions of about 3·3 ml. by means of an automatic fraction collector.

In the majority of experiments only 450 ml. of eluate (which contained most of the amino acids) was collected; no attempt was usually made to recover arginine and phenylalanine, which were present in the last 150 ml. to emerge from the column. Preliminary experiments revealed no significant radioactivity in this part of the eluate.

Detection of amino acids in the eluate. Amino acids were detected by the ninhydrin method described by Chibnall, Mangan & Rees (1958). A sample (0·2 ml.) of each fraction was neutralized with NaOH and diluted with water to 2 ml. To this was added 1 ml. of a buffer made up as follows. To 23 ml. of 5 M-sodium acetate buffer (pH 5·5) was added 2 ml. of 0·1 M-KCN and 75 ml. of 2-methoxyethanol (methyl Cellosolve; Hopkin and Williams Ltd.). Ninhydrin (0·2 ml. of 10%, w/v, solution in 2-methoxyethanol) was then added and the mixture heated in a boiling-water bath for 20 min. The mixture was cooled, diluted to 10 ml. with 60% ethanol and the light absorption at 570 mμ measured in a spectrophotometer.

Detection of ^{14}C *in the eluate.* The remaining portion of each fraction was evaporated to dryness *in vacuo*, redissolved in 0·5 ml. of water, transferred to an aluminium disk (2 cm. diameter) and dried as a thin film by the method of Boursnell, Coombs & Rizk (1953). Fractions containing no amino acid, or amino acid expected to have no ^{14}C, were combined and similarly treated. The radioactivity was measured beneath a thin end-window Geiger–Müller tube over a period of 20 min. Counts/min. ranged between 0 and 1000.

RESULTS

The order of elution from the column of those amino acids with which we were principally concerned was aspartic acid, serine + threonine, glutamic acid, glycine and alanine. All the other amino acids emerged after these. It was not possible to distinguish between serine and threonine. The peak for serine + threonine to some extent overlapped that for aspartic acid, but it was possible to distinguish between the ^{14}C contained in the aspartic acid fraction and that in the serine + threonine fraction, from the shape of the peak of the curve and the position of its maximum point. Likewise, some overlap between the glycine and alanine fractions occurred, but it was possible similarly to determine whether ^{14}C was contained in the alanine or in the glycine fraction. No ^{14}C was detected in any fraction other than those considered below.

When protein from isolated rat diaphragm which had been incubated in a medium containing D-[U-^{14}C]glucose was hydrolysed and the amino acids were separated, ^{14}C was found to be present in the fractions containing aspartic acid and glutamic acid and alanine (Table 1), the greatest part being in the alanine fraction. With diaphragm incubated with [1-^{14}C]pyruvic acid, ^{14}C occurred in the fractions of the protein hydrolysate which contained aspartic acid and glutamic acid and alanine, the major portion of the activity again being in the alanine fraction. When diaphragm was incubated with a mixture of L-[U-^{14}C]glutamic acid and [5-^{14}C]oxoglutaric acid, under conditions in which Manchester & Young (1958*b*) found that 75% of the ^{14}C incorporated into protein was derived from the oxoglutaric acid, ^{14}C was found chiefly in glutamic acid and to a less extent in aspartic acid (Table 1). Analysis of the amino acids obtained by hydrolysis of protein from diaphragm incubated with $NaH^{14}CO_3$ revealed ^{14}C to be present in the fractions containing aspartic acid and glutamic acid to an approximately equal extent (Table 1).

Because these observations suggested that a significant rate of synthesis of amino acids from non-amino acid sources occurred in diaphragm under the conditions of our experiments, it was of

Table 1. *Location of* ^{14}C *in protein obtained from rat-diaphragm muscle which had been incubated in vitro with* ^{14}C*-labelled substances*

Diaphragm incubated with	Total counts/min. eluted from column	Radioactivity in hydrolysate from 10 mg. of protein: Percentage of ^{14}C appearing in: Aspartate	Serine + threonine	Glutamate	Glycine	Alanine	Other amino acids
D-[U-^{14}C]Glucose	185	17	0	24	0	59	0
*D-[U-^{14}C]Glucose	227	15	0	28	0	57	0
[1-^{14}C]Pyruvate	214	14	0	10	0	76	0
L-[U-^{14}C]Glutamate and [5-^{14}C]oxoglutarate	142	28	0	72	0	0	0
$NaH^{14}CO_3$	314	48	0	52	0	0	0
L-[U-^{14}C]Alanine	2168	6	0	12	0	82	0
*L-[U-^{14}C]Alanine	3232	7	0	16	0	77	0
L-[U-^{14}C]Glutamate	1535	23	0	74	0	3	0
L-[U-^{14}C]Aspartate	1425	43	0	42	0	15	0
[1-^{14}C]Glycine	1436	0	6	0	94	0	0
*[1-^{14}C]Glycine	2194	0	7	0	93	0	0
L-[U-^{14}C]Serine	1567	0	86	0	14	0	0

* Insulin (0·5 unit/ml.) was added to the medium.

interest to investigate the extent to which certain amino acids might be broken down and converted into other amino acids under these conditions. When protein from isolated diaphragm which had been incubated in a medium containing L-[U-^{14}C]-alanine was hydrolysed, and the amino acids were separated, ^{14}C was found to be present largely in alanine but also to a less extent in aspartic acid and glutamic acid (Table 1). Incubation of diaphragm with L-[U-^{14}C]glutamic acid led to a recovery of ^{14}C chiefly in glutamic acid, but also to a less extent in aspartic acid, with a very little in alanine (Table 1). Incubation with L-[U-^{14}C]aspartic acid led to an almost equal division of ^{14}C between aspartic acid and glutamic acid, with a smaller but appreciable amount in alanine (Table 1).

With [1-^{14}C]glycine most of the ^{14}C recovered was found in the glycine fraction of the protein from diaphragm but a small proportion occurred in the serine + threonine fraction (Table 1). Incubation of diaphragm with L-[U-^{14}C]serine led to a recovery of ^{14}C almost entirely in the serine + threonine fraction, but a small proportion of ^{14}C was also found in the glycine fraction (Table 1).

All these observations were made with a medium containing no added insulin. Addition of insulin (0·5 unit/ml.) to the medium when diaphragm was incubated with [^{14}C]glycine, [^{14}C]alanine or [^{14}C]-glucose, raised the amount of ^{14}C incorporated into protein of the diaphragm by 25–50% but in no case was the distribution of ^{14}C amongst the amino acids of the protein hydrolysate changed (Table 1).

The results are summarized in Fig. 1.

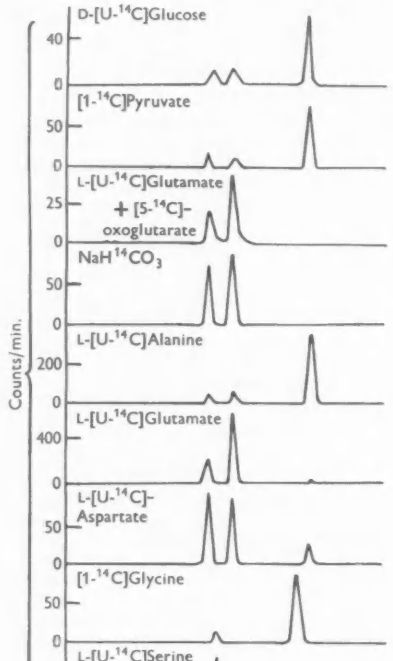

Fig. 1. Location of [^{14}C]amino acids in hydrolysates from protein obtained from isolated rat diaphragm incubated *in vitro* with [^{14}C]amino acids. The amino acids were separated by column chromatography (see text).

DISCUSSION

Insulin and incorporation of amino acids. Manchester & Young (1958*a*) found that with isolated rat diaphragm incubated *in vitro* incorporation into protein of radioactive material from all the 11 labelled amino acids tested was stimulated by addition of insulin to the medium. Except with alanine, this stimulating action of insulin was little affected by the addition of glucose or of pyruvic acid to the medium. The action of insulin in stimulating the incorporation of ^{14}C from [^{14}C]-alanine into diaphragm protein was abolished by the addition of glucose to the medium. This atypical behaviour of [^{14}C]alanine, and the observation, again peculiar to alanine, that addition of glucose or of pyruvic acid to the medium in the absence of insulin depressed incorporation of ^{14}C from the amino acid into diaphragm protein, was explicable by a dilution of labelled alanine with unlabelled alanine formed from the glucose or pyruvic acid added (Manchester & Young, 1958*b*). It was therefore not necessary to postulate an action of insulin on the incorporation of alanine qualitatively different from that of insulin on the incorporation of other amino acids.

In the absence of evidence to the contrary it is reasonable to assume that insulin stimulates incorporation of radioactive material from labelled amino acids into protein of diaphragm by a mechanism that is similar for all amino acids. This assumption is supported by the observation reported here, that addition of insulin to the medium when diaphragm was incubated with [^{14}C]glycine, [^{14}C]alanine or [^{14}C]glucose raised the total in-

corporation but did not affect the distribution of ^{14}C among the amino acids of the protein. Since the rise in incorporation of ^{14}C from glucose induced by insulin was 23%, whereas the rise in ^{14}C from alanine and glycine with insulin was 49 and 53% respectively, the effect of the hormone on the incorporation of amino acids by diaphragm *in vitro* is of a magnitude at least similar to that of its effect on glucose utilization. These observations provide evidence against the idea that, in influencing protein biosynthesis, insulin acts by stimulating reactions specific for the metabolism of only a limited number of amino acids, or for that of substances involved in glycolysis. They agree with the view that insulin stimulates protein biosynthesis by promoting the transfer of amino acids across the cell membrane, but provide no direct evidence for it.

Fixation of carbon dioxide. Manchester & Young (1958*b*) found that addition of [^{14}C]glucose or of [^{14}C]pyruvic acid to the medium in the presence of [^{14}C]alanine results in no diminution of incorporation of ^{14}C into protein of isolated rat diaphragm such as normally occurs when unlabelled glucose or pyruvate is added to the medium. This observation suggested that both glucose and pyruvic acid can rapidly provide the carbon skeleton of alanine in isolated rat diaphragm *in vitro*. We have now confirmed this suggestion by finding that the principal [^{14}C]amino acid formed from [U-^{14}C]-glucose or [1-^{14}C]pyruvic acid is alanine, although indeed some labelling of the aspartic acid and glutamic acid occurred. The appearance of [^{14}C] in aspartic acid and glutamic acid under these conditions is of particular interest, because on the basis of present views it would be expected that if the carbon skeletons of these amino acids are formed from [1-^{14}C]pyruvic acid by the action of pyruvic dehydrogenase together with the operation of the tricarboxylic acid cycle, the amino acids thus made would be unlabelled (see Krebs, 1954). It is possible, however, that the [^{14}C]-aspartic acid and -glutamic acid are formed by processes which involve fixation of $^{14}CO_2$ into protein of diaphragm (Manchester & Young, 1958*b*), the $^{14}CO_2$ being formed by the decarboxylation of [1-^{14}C]pyruvic acid by pyruvic dehydrogenase. If this is so, $^{14}CO_2$ produced by the action of pyruvic dehydrogenase on [1-^{14}C]pyruvic acid must be trapped in some way in our experiments before it has time to equilibrate with the unlabelled carbon dioxide and HCO_3^- ions in the diaphragm and buffer. Bachhawat & Coon (1957) have suggested that carbon dioxide used in carboxylation first forms a complex with adenylic acid; in this way the carbon dioxide liberated from pyruvic acid by pyruvic dehydrogenase may be trapped. On the other hand, if pyruvic dehydrogenase is located in the mitochondrion (Vennesland, 1951), and the mitochondrion is not very permeable to carbon dioxide, the trapping of a significant proportion of the carbon dioxide might also be expected.

Manchester & Young (1958*b*) suggested that ^{14}C from $^{14}CO_2$ which enters the protein in diaphragm does so through a fixation of carbon dioxide by pyruvic acid to form oxaloacetic acid (Krebs & Eggleston, 1940), which is then aminated to form aspartic acid. If this were so, ^{14}C should be recoverable from the aspartic acid fraction of the protein hydrolysate, and this has now been done. Although Stern (1948) failed to find direct evidence for the presence in muscle of a reversible oxaloacetic decarboxylase, our results can be easily and simply explained on the basis of the presence of such an enzyme system in rat-diaphragm muscle. On the other hand, as much ^{14}C is found in glutamic acid as in aspartic acid in our experiments, and although this might be explained by interconversion of aspartic acid and glutamic acid through the tricarboxylic acid cycle, a possibility exists that a second way of fixing carbon dioxide can function in muscle. Such a way might be by fixation of carbon dioxide by succinyl-coenzyme A or bound succinic semialdehyde to form oxoglutaric acid, which would then be aminated to form glutamic acid (Kaufman, 1951). It has not been shown that the decarboxylation of oxoglutaric acid to succinyl-coenzyme A and carbon dioxide can be reversed, but since Ochoa (1945) has found that the enzymic decarboxylation of oxalosuccinic acid to oxoglutaric acid and carbon dioxide is reversible, there is no reason to suppose that the former reaction cannot also be reversed.

When $NaH^{14}CO_3$ is incubated with diaphragm similar amounts of $^{14}CO_2$ enter aspartic acid and glutamic acid. Furthermore, the incubation of diaphragm with [U-^{14}C]aspartic acid leads to as much incorporation of ^{14}C into glutamic acid as into aspartic acid, with a small incorporation into alanine also. We believe that since this transfer of ^{14}C from aspartic acid to glutamic acid is quite large, it is more probably a result of operation of the tricarboxylic acid cycle than a fixation by succinyl-coenzyme A into oxoglutaric acid of $^{14}CO_2$ released from oxaloacetic acid (formed from aspartic acid) during the operation of the tricarboxylic acid cycle. Nevertheless, our experiments do not at present provide decisive evidence by which a distinction between the various possible interpretations we have considered may be made.

Hiatt, Goldstein, Lareau & Horecker (1958) have recently found that when isolated diaphragm is incubated with $NaH^{14}CO_3$, ^{14}C is incorporated into glycogen. They considered the possibility that ^{14}C may become incorporated into phosphopyruvic acid formed from pyruvic acid by operation of the

'dicarboxylic acid shuttle' (Ochoa, Mehler & Kornberg, 1948; Utter & Kurahashi, 1953), but concluded that these reactions, though occurring rapidly in liver, take place to only a small extent if at all in muscle. They therefore considered the possibility of an exchange of the pyruvic acid carboxyl group with $^{14}CO_2$ in the pyruvic acid oxidation reaction demonstrated with a mammalian enzyme system by Goldberg & Sanadi (1952). We have detected no ^{14}C in alanine, and our results therefore provide no support for this view.

On the other hand, Goldberg & Sanadi (1952) found that ^{14}C could become incorporated into oxoglutaric acid when the latter reacts with $NaH^{14}CO_3$ in the presence of α-oxoglutaric oxidase prepared from pig heart. The occurrence of such a reaction in diaphragm would explain the entry of ^{14}C into glutamic acid in diaphragm protein which we have observed when $NaH^{14}CO_3$ is incubated with isolated rat diaphragm.

Metabolism of amino acids. In the studies with [^{14}C]amino acids, ^{14}C from [^{14}C]alanine enters the glutamic acid and aspartic acid fractions to a surprising extent, and the transfer of ^{14}C from [^{14}C]glutamic acid to [^{14}C]aspartic acid is also large. A substantial transfer of ^{14}C from [^{14}C]aspartic acid to [^{14}C]alanine has also been observed, presumably through the decarboxylation of oxaloacetic acid to pyruvic acid, the oxaloacetic acid being derived from the added aspartic acid. A smaller transfer of ^{14}C to alanine from [^{14}C]glutamic acid could be a consequence either of the presence at the site of action of a smaller concentration of endogenous aspartic acid than is the case when this substance is added to the medium, or of the location of oxaloacetic decarboxylase outside of the mitochondrion associated with a formation of aspartic from glutamic acid within the mitochondrion.

Though all the observations reported here cannot easily be explained in terms of known processes, it is clear that in isolated diaphragm rapid operation of the tricarboxylic acid cycle takes place, together with interconversion of aspartic acid, glutamic acid and alanine and their corresponding keto acids.

Though the major portion of ^{14}C incorporated into diaphragm protein from [^{14}C]glycine is contained in the glycine fraction, some conversion of the labelled glycine into serine (or into constituents of the serine + threonine fraction) appears to occur. Similarly, though most of the ^{14}C from [^{14}C]serine can be recovered in the serine + threonine fraction, some formation of labelled glycine from the serine appears to take place. The interconversion of serine and glycine has been demonstrated previously in liver (Siekevitz, Winnick & Greenberg, 1949; Vilenkina, 1952), but not in muscle.

SUMMARY

1. Isolated rat diaphragm has been incubated with various [^{14}C]amino acids and metabolites, and protein from the diaphragm has been hydrolysed and separated by column chromatography into its component amino acids.

2. When diaphragm is incubated with [U-^{14}C]-glucose or with [1-^{14}C]pyruvic acid, ^{14}C is incorporated mainly into the alanine fraction and to a less extent into aspartic acid and glutamic acid.

3. Incubation of diaphragm in a medium containing $^{14}CO_2$ leads to a recovery of similar quantities of ^{14}C in aspartic acid and glutamic acid.

4. Diaphragm incubated with [U-^{14}C]alanine incorporates ^{14}C mainly into the alanine fraction of the protein, though some ^{14}C is also found in aspartic acid and glutamic acid. Incubation with [U-^{14}C]aspartic acid gives rise to ^{14}C in the glutamic acid and alanine fractions as well as in aspartic acid, whereas incubation with [U-^{14}C]glutamic acid leads to an appreciable recovery of ^{14}C in aspartic acid and glutamic acid alone.

5. ^{14}C incorporated into protein of diaphragm from [1-^{14}C]glycine is recovered almost entirely as [^{14}C]glycine, the remainder being found in the serine + threonine fraction. Conversely, protein of diaphragm incubated with [U-^{14}C]serine contains ^{14}C mainly in the serine + threonine fraction but also to a small extent in the glycine fraction.

6. Addition of insulin to the medium, although increasing the amount of ^{14}C incorporated by 25–50%, did not change the distribution of ^{14}C incorporated from [^{14}C]glucose, alanine and glycine.

7. The results are largely explicable in terms of rapid transaminations and operation of the tricarboxylic acid cycle in isolated rat diaphragm, together with incorporation of carbon dioxide by pyruvate, succinyl-coenzyme A or related substances.

We wish to express our thanks to Mr M. W. Rees for much helpful advice on the practice of column chromatography and for the loan of one of the columns used in this work. This work has been greatly helped by a generous grant from the British Diabetic Association for the purchase of radioactive amino acids. The Medical Research Council provided a personal grant for K.L.M. To the British Diabetic Association and to the Medical Research Council we wish to express our thanks for assistance in this way, which has made this research possible.

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The Reactions of Amino Acids with Soluble Ribonucleic Acid from Guinea-Pig Mammary Cells

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During the last 5 years many cell-free systems of animal, plant and bacterial origin have been prepared which were able to perform some of the intermediate reactions which are likely to be on the pathway to protein synthesis. The mammary gland in full lactation is a most suitable system for the study of every aspect of protein synthesis; not only are large quantities of protein continuously formed but they are also excreted from the cells and thus the complications introduced by catabolism are avoided. Barry (1958) has described various studies of the formation of milk proteins from injected labelled amino acids *in vivo*. He concluded that milk proteins are formed from free amino acids of the blood stream. The development of cell-free preparations from liver, which could incorporate [^{14}C]amino acids into the peptide linkage of microsomal protein (Zamecnik & Keller, 1954; Keller & Zamecnik, 1956), led to the discovery of three distinct steps in this process (Hoagland, Stephenson, Scott, Hecht & Zamecnik, 1958). The three steps are: first, the activation of amino acids by the enzymic formation of amino acid adenylates; secondly, the combination of activated amino acids with cell-sap ribonucleic acid; and thirdly the transfer of amino acids from cell-sap ribonucleic acid to microsomal nucleoprotein.

In this Laboratory we have studied many aspects of amino acid incorporation with a number of different systems prepared from lactating mammary glands. The pattern of incorporation of amino acids into subcellular particles and soluble protein of intact mammary cells and tissue homogenates *in vitro* has been described recently (Fraser & Gutfreund, 1958), and we have shown that the three steps studied by Hoagland *et al.* (1958) can be usefully investigated in mammary-tissue homogenates. We have now studied the formation of cell-sap ribonucleic acid–amino acid compounds in greater detail. Our results do not enable us to draw any conclusions about the chemical mechanism of this reaction, but they give a quantitative picture of the proportions of different amino acids involved, of their competition with each other and of the rates and equilibria of the compound formation. It will be seen that certain conclusions can be drawn from such data about the role of cell-sap ribonucleic acid–amino acid coupling in the overall process of protein synthesis.

EXPERIMENTAL

Materials. All [^{14}C]amino acids were obtained from The Radiochemical Centre, Amersham, Bucks. Their specific activities are quoted in relevant places in the text. The L-amino acids are generally labelled, and the DL-amino acids are [1-^{14}C]. The adenosine triphosphate (ATP) used

was obtained from L. Light and Co. Ltd., Colnbrook, Bucks, as the free acid and converted into the magnesium dipotassium salt (MgK_2ATP).

Preparation of cell fractions. Guinea pigs, within 2–7 days after parturition, were killed by a blow on the head. Their mammary glands were excised and plunged into ice-cold medium A (containing 0·35 M-sucrose, 0·035 M-$KHCO_3$, 0·025 M-KCl, 0·01 M-$MgCl_2$; pH 7·4), after Keller & Zamecnik (1956). After cooling for 3–4 min. the glands were dried and freed of extraneous fat, muscle and connective tissue and minced. The mince was weighed and 2·3 vol./wt. of ice-cold medium A added with stirring. It is important to keep the tissue cold in any operation subsequent to excision of the glands. The mince suspension was then homogenized in a Potter homogenizer kept in ice. The cell fractions were separated by differential centrifuging in a refrigerated Spinco preparative ultracentrifuge at 0°. An initial spin for 10 min. at 10 000 *g* sedimented the debris, nuclei and mitochondrial fractions. The 'microsome-rich fraction' thus obtained was then diluted 1:1 (v/v) with ice-cold medium A. A second spin for 90–120 min. at 105 000 *g* sedimented the microsome fraction, leaving a clear-pink supernatant fraction, which was carefully pipetted off.

Preparation of the 'pH 5 fraction' and cell-sap ribonucleic acid. The supernatant fraction from the differential centrifuging was further diluted 1:1 (v/v) with ice-cold 0·15 M-KCl and adjusted to pH 4·9 with N-acetic acid. The solution became turbid and the precipitate, the 'pH 5 fraction', was spun down, washed once by resuspending in ice-cold 0·15 M-KCl and again centrifuged. The final precipitate was then redissolved ready for use in a volume of medium A equal to the weight of the minced tissue used. In this way we obtained a suspension of 'pH 5 fraction' which contained 10–15 mg. of protein/ml. and 0·3–0·4 mg. of cell-sap ribonucleic acid (RNA)/ml. From this pH 5 fraction cell-sap RNA was prepared by the phenol-extraction procedure of Kirby (1956) as modified by Hoagland *et al.* (1958).

Estimation of cell-sap ribonucleic acid. The concentration of cell-sap RNA in the pH 5 fraction was determined after the method of Hoagland *et al.* (1958), by digestion of a sample in N-NaOH for 1 hr. at 37°, precipitation of the protein with 5 N-HCl and determination of the extinction at 260 mμ with a Unicam model SP. 500 spectrophotometer. An extinction coefficient of 34·2 $mg.^{-1}$ $cm.^2$ was used to calculate the cell-sap RNA concentration.

Procedure for labelling cell-sap ribonucleic acid by [^{14}C]amino acid–cell-sap ribonucleic acid coupling

Labelling experiments. Incubation mixtures contained MgK_2ATP, 'pH 5 fraction' dissolved in medium A (see above), medium A (with or without dissolved carrier or competitor amino acids) and [^{14}C]amino acid, in the ratio of 2:10:7:1 (by vol.), the final concentrations of ATP and [^{14}C]amino acid being respectively 0·01 M and 0·5 μc/ml., except where stated. The total volume was adjusted according to the needs of the experiment. Incubations were carried out aerobically in a water bath at 37°. At appropriate times samples (1 or 2 ml.) were taken and precipitated in 5 ml. of ice-cold 10% trichloroacetic acid containing 1% of carrier amino acid. Either the total pH 5 fraction (cell-sap RNA + protein) or the pH 5 protein was then isolated for counting.

'Delabelling' experiments. The pH 5 fraction was prelabelled as above for 30 min. The reaction was stopped by plunging the flask into an ice bath with efficient stirring of the reaction mixture. The pH 5 fraction was then precipitated again by adjusting the pH to 4·9 with N-acetic acid and washed with ice-cold 0·15 M-KCl. The labelled pH 5 fraction was redissolved in the original volume of fresh cold medium A, with appropriate amounts of added MgK_2ATP, as described. Further incubation was then carried out for 'delabelling' at 37°, taking appropriate time samples (1 or 2 ml.) for precipitation in ice-cold 10% trichloroacetic acid containing 1% of carrier amino acid.

Preparation of samples for counting

The total pH 5 fraction (cell-sap RNA + protein) was isolated from the trichloroacetic acid precipitates (see above) by a slight modification of the method of Hoagland *et al.* (1958). The precipitates were washed twice with cold 5% trichloroacetic acid containing 1% of carrier, and once each with 80% ethanol, acetone, absolute ethanol–ether (3:1, v/v) and ether, in that order. The samples were then air-dried for counting. Cell-sap RNA as well as [^{14}C]amino acid-labelled cell-sap RNA samples were prepared in the same way.

Determination of radioactivity

All samples were counted at infinite thickness, on 0·3 $cm.^2$ or 1·0 $cm.^2$ plastic planchets. A [^{14}C]Perspex standard (supplied by The Radiochemical Centre) was used for frequent standardization of the counter. All samples were counted on a Tracer-Lab internal gas-flow counter. The background count varied from 16 to 18 counts/min. All counts are recorded as counts/min./$cm.^2$ at infinite thickness, corrected for background. From self-absorption experiments carried out over a wide range of sample weights the relation counts/min./mg. = counts/min./$cm.^2$ (infinite thickness)/2·2 was established.

RESULTS

Apart from the previously noted isolation of [^{14}C]-amino acid-labelled cell-sap RNA two types of experiment were carried out to show that the significant part of the ^{14}C-labelling of pH 5 fraction on incubation with ATP and [^{14}C]amino acids is due to the coupling of amino acids to the cell-sap RNA of this fraction. Fig. 1 shows the time course of the combination of [^{14}C]leucine with total pH 5 fraction and with protein prepared from this fraction. As indicated in the section above there is about 30 times as much protein as nucleic acid in the pH 5 fraction. We have confirmed the requirement for ATP for this reaction in the mammary-gland system, which has been reported by Hoagland *et al.* (1958) for experiments with rat-liver pH 5 fraction.

Next we investigated the effect of amino acid uptake into the pH 5 fraction when additional separately prepared cell-sap RNA is added. We found that even if rat-liver cell-sap RNA is added to guinea-pig mammary gland pH 5 fraction the amount of coupled amino acid is proportional to the

total cell-sap RNA concentration. The results of one such experiment are recorded in Fig. 2.

The experiments recorded in Figs. 3 and 4 were carried out to study the dependence of the extent of cell-sap RNA–amino acid combination on amino acid concentration. Unlabelled amino acids were added to make up the total concentration, and though DL-amino acids were used the concentrations of L-amino acids are given and the counts are divided by the specific activities of the amino acid preparations used. A separate experiment with glycine, extended to a concentration of 0·02 M, showed that the increase in cell-sap RNA–glycine combination remained linear.

When the time course of cell-sap RNA–amino acid combination is followed one can always observe a reversal of this process after incubation for about 30 min. (see Fig. 1). We therefore incubated [^{14}C]amino acid-labelled, precipitated and washed pH 5 fraction, which had been redissolved in medium A, in the presence of 0·01 M-ATP, as indicated above. Fig. 5*A* shows the results of such a delabelling experiment carried out with [^{14}C]-leucine-labelled pH 5 fraction. The reversibility of the removal of leucine was tested in a parallel experiment (Fig. 5*B*) in which [^{14}C]leucine was added 15 min. after the beginning of incubation in the amino acid-free medium. To determine whether the time course of the removal of leucine from its compound with pH 5 fraction was the same when the compound formation occurred at relatively high leucine concentration, pH 5 fraction, pre-labelled with 0·01 M-leucine, was subsequently incubated in an amino acid-free medium as above (Fig. 5*C*). This removal of amino acids from the compound was shown to be quite independent of the presence or absence of ATP, whereas there was the same requirement for ATP for the reversal of this process as for the initial compound formation.

Fig. 6 shows the results of experiments on the comparative effect of L-[^{14}C]leucine and DL-[^{14}C]-leucine on the labelling of pH 5 fraction. The total concentration of leucine was the same (80 μM) in both experiments. The counts obtained from the samples of the experiment with DL-leucine were multiplied by 0·66/0·41 to correct for the greater specific activity of the L-leucine preparation.

Two experiments to investigate competitive inhibition of amino acids for their combination with pH 5 fraction are recorded in Fig. 7. As can be seen, there is some inhibition of leucine coupling in the presence of high concentrations of valine, whereas there is no inhibition of valine coupling in the presence of high concentrations of leucine. Similarly, we found that glycine (M) did not reduce the final amount of valine combined with pH 5 fraction, though it did slow down the rate of coupling process.

Two types of experiment were carried out to investigate whether the reactions of glutamic acid and glutamine with pH 5 fraction are independent of each other. DL-[^{14}C]Glutamic acid (0·25 μC/ml.), ATP (0·01 M) and pH 5 fraction were incubated for

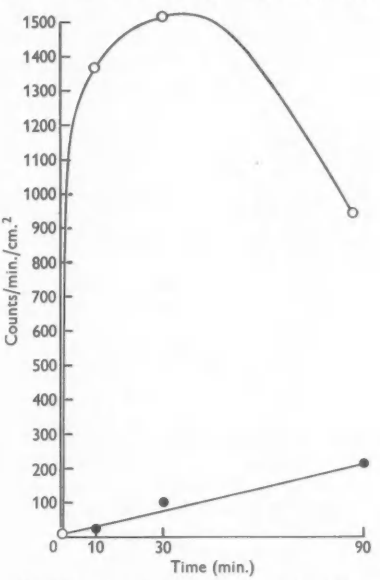

Fig. 1. Combination of L-[^{14}C]leucine with total pH 5 fraction (○) and with the protein of pH 5 fraction (●). Incubation was as described in the text; [^{14}C]leucine 57 μM, 0·5 μC/ml.

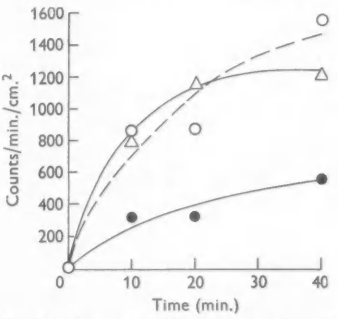

Fig. 2. Combination of [^{14}C]glycine with guinea-pig mammary gland pH 5 fraction (●; 0·35 mg. of cell-sap RNA/ml.) and the effect of added (0·65 mg./ml.) rat-liver cell-sap RNA (Δ) on glycine uptake by this fraction. Incubation mixture was as described in the text; [^{14}C]glycine 50 μM, 0·25 μC/ml. The broken line (○) is calculated for the expected uptake if this is proportional to increase in cell-sap RNA concentration.

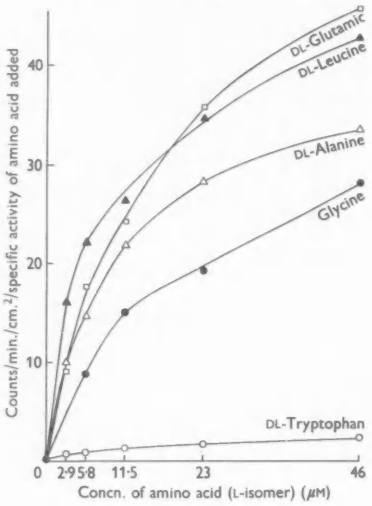

Fig. 3

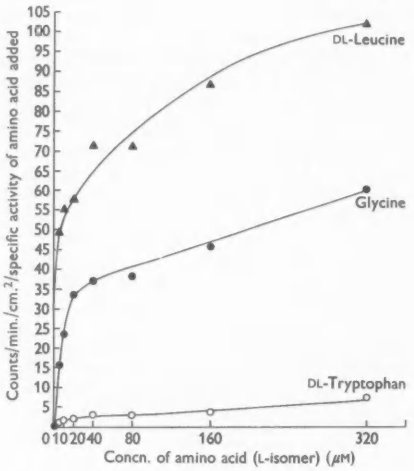

Fig. 4

Figs. 3, 4. Combination of several amino acids with pH 5 fraction over a range of amino acid concentration. Incubation was for 30 min. as described in the text.

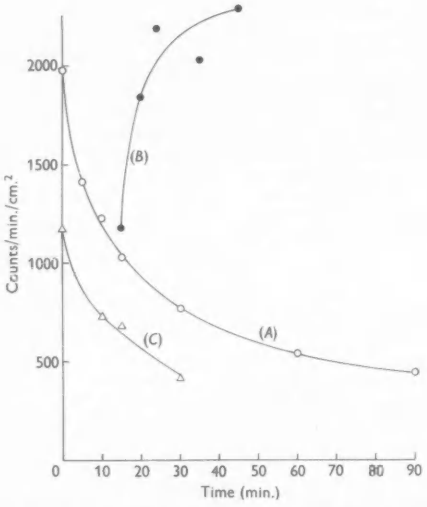

Fig. 5. Removal of [^{14}C]leucine from its compound with pH 5 fraction in amino acid-free medium: (*A*) labelled with L-leucine (50 μM, 0·37 μc/ml.) for 30 min. before incubation; (*B*) [^{14}C]leucine (0·3 μc/ml.) added after 15 min.; (*C*) labelled with L-leucine (10 mM, 1 μc/ml.) for 30 min. before incubation.

10 min. (1) without any addition, (2) in the presence of unlabelled L-glutamic acid (0·01 M), (3) in the presence of unlabelled L-glutamine (0·01 M) and (4) in the presence of Elliott's (1953) glutamine synthetase system. The counts/min./cm.² obtained for samples of pH 5 fraction subsequently prepared from the above four incubation mixtures in the usual manner were 104, 0, 32 and 210 respectively. Though lack of stability of glutamine caused some dilution of glutamic acid label in (3), the relatively small dilution at such a large carrier concentration indicates separate specificity for glutamic acid and glutamine. The increase of labelling of pH 5 fraction in the presence of [^{14}C]glutamine and [^{14}C]glutamic acid in incubation (4) corroborates this idea.

DISCUSSION

The experiments described in this paper were designed to investigate quantitative aspects of the reactions of cell-sap RNA with amino acids, with the view of elucidating the particular functions of these reactions in the overall process of protein biosynthesis. The mechanism of the condensation of amino acids to peptide chains for the formation of protein molecules has to fulfil two tasks. First, it has to couple the endergonic peptide-bond forma-

tion with an exergonic reaction to make the overall reaction thermodynamically possible. Secondly, it has to include a step or series of steps which determine the specific sequence in which the amino acids condense. The first part of this problem has been solved in principle by the discovery of enzyme systems which catalyse the formation of the high-energy amino acid adenylates from amino acids and ATP. It is widely believed that specific amino acid-activating enzymes will be found for each individual amino acid; that it has not yet been shown whether there are 20 distinct activating enzymes or fewer enzyme systems capable of activating all 20 amino acids is probably due to the use of inadequate methods of fractionation and enzymic assay of the particle-free supernatant. As anticipated, preliminary studies of amino acid activation in mammary tissue (Gutfreund, 1958; Fraser & Gutfreund, 1958) gave results which have to be revised, and far more detailed investigations of these enzyme preparations and reactions are in progress in this Laboratory.

Since it was shown by Hoagland *et al.* (1958) that the reactions of amino acids with cell-sap RNA occur after the enzymic formation of amino acid adenylates and before the incorporation of the amino acids into protein in the microsomal particles, one would like to find any possible role of cell-sap RNA in the determination of the specific sequence. It was reported by Fraser & Gutfreund (1958) that the relative number of specific places available on cell-sap RNA for different amino acids appears to bear some relation to their abundance in the protein synthesized. The more detailed experiments with six amino acids described in this paper confirm this point. For most of our cell-sap RNA–amino acid-coupling experiments we took as a measure of the quantities of [^{14}C]amino acids bound to cell-sap RNA the radioactivity of suitably prepared specimens of the pH 5 fraction, which contained protein as well as RNA. We have carried out a number of experiments to separate cell-sap RNA and protein of amino acid-labelled pH 5 fraction. Fig. 1 shows the relative labelling of cell-sap RNA and protein under a variety of conditions. It seems likely that the combination of protein with [^{14}C]amino acids is due to a non-specific reaction of activated amino acids, which is further discussed below. We have also shown that the addition of cell-sap RNA to pH 5 fraction increases the uptake of amino acids on incubation and that this uptake is proportional to total cell-sap RNA concentration. As shown in Fig. 2, there is a proportional increase in amino acid uptake when cell-sap RNA prepared from rat liver is added to

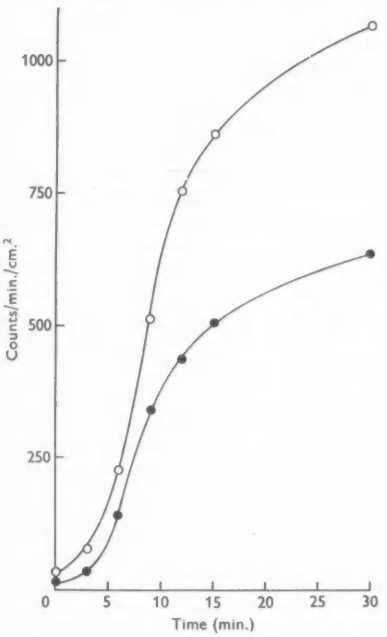

Fig. 6. Combination of DL-[^{14}C]leucine (●; 0·41 μc/ml.) and L-[^{14}C]leucine (○; 0·66 μc/ml.); with pH 5 fraction. Total leucine concentration in each case was 80 μM.

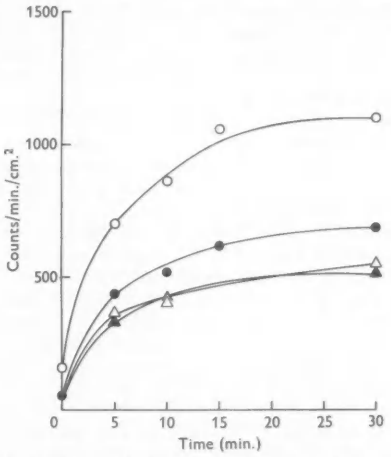

Fig. 7. Inhibition of the combination of DL-leucine by DL-valine. Concentrations of [^{14}C]amino acids and unlabelled amino acids in the four incubation mixtures were as follows: ○, DL-[^{14}C]leucine, 98 μM; ●, DL-[^{14}C]-leucine, 98 μM, and unlabelled DL-valine, 0·318 M; △, DL-[^{14}C]valine, 0·28 mM; ▲, DL-[^{14}C]valine, 0·28 mM, and unlabelled L-leucine, 0·15 M.

pH 5 fraction from guinea-pig mammary gland. It would, of course, be of great interest to have precise data from the number of nucleotides per binding site for each individual amino acid. The evaluation of such figures is complicated by the form of the saturation curves for cell-sap RNA–amino acid coupling. A first inspection of the plots of amino acid concentration against a measure of the bound amino acid residues (Figs. 3, 4) shows that the process is biphasic. First there is a rapid increase of cell-sap RNA–amino acid compound formation with increasing concentration of amino acids up to about 20 μM, and then there follows a slower continuous increase in compound formation proportional to amino acid concentration. One can calculate from our data for some amino acids at high concentrations (about 0·1 M) that they combine to the extent of one amino acid residue per nucleotide. It appears likely that the cell-sap RNA–amino acid combination at low amino acid concentration involves occupation of sites specific for each amino acid, whereas the extensive further combination involves the non-specific reaction of activated amino acid adenylates with hydroxyl and other groups of cell-sap RNA. That such non-specific reactions occur has been demonstrated by Castelfranco, Moldave & Meister (1958).

Two different experiments were carried out to investigate the properties of cell-sap RNA–amino acid compounds formed at relatively high amino acid concentration in an attempt to distinguish the linkages thus formed from those which are thought to be on the specific sites. We found that incubation of the total pH 5 fraction with ATP and M-glycine or 0·15M-leucine had no significant effect on the combination of cell-sap RNA with valine. It appears therefore that the specific places for individual amino acids are still available even if very extensive combination with what are assumed to be non-specific sites has taken place. Another property of extensively coupled cell-sap RNA is that the amino acids are removed from it under the same conditions and at the same rate as from the specific cell-sap RNA–amino acid compounds. We have studied the time course and conditions of this 'delabelling' process, and though we have little information about the chemical mechanism involved, our data can be correlated with chemical investigations carried out in other laboratories. Fig. 5 shows that if [^{14}C]leucine-labelled pH 5 fraction precipitated at pH 4·9 is resuspended in medium A and 0·01M-ATP, leucine is rapidly removed from the compound. It has been shown that this process is reversible since on further addition of leucine after 15 min. the compound is reformed (Fig. 5*B*). Preliminary experiments indicate that at least some of the leucine liberated is associated with material absorbing at 260 mμ.

In view of the experiments reported by Hecht, Stephenson & Zamecnik (1958) and by Zachau, Acs & Lipmann (1958), it seems to us reasonable to suggest that the enzymic labelling of cell-sap RNA is reversed by enzymic removal of an amino acid-adenosine monophosphate compound. When there is an ample supply of free amino acids and ATP in solution the rate of compound formation is considerably greater than the reverse process. This reversal also occurred in the absence of ATP and is probably due to ribonuclease. A reversal of cell-sap RNA–amino acid compound formation, which is probably of a different nature, was reported by Glassman, Allen & Schweet (1958), who found that if the compound is incubated with activating enzyme, [^{14}C]adenosine monophosphate and pyrophosphate in the absence of free amino acids and ATP, then free amino acids and [^{14}C]ATP are produced.

It has been mentioned above that incubation of pH 5 fraction with glycine or leucine does not interfere with cell-sap RNA–valine compound formation. We have been able to show, however, that some competition between amino acids can occur. Large concentrations of valine, for instance, will interfere with the cell-sap RNA–leucine combination (Fig. 7). One can speculate here that of the two structurally similar amino acids, leucine and valine, the smaller one will fit into the site of the larger one but not vice versa.

As indicated in the description of individual experiments, data for the quantitative aspects of the specificity of cell-sap RNA–amino acid combination were obtained by the use of DL-[^{14}C]amino acids. Synthetic DL-amino acids are much less likely to be contaminated with other amino acids than the natural L forms, which are prepared chromatographically. A comparison of the cell-sap RNA–leucine combination during incubation of pH 5 fraction, ATP and L-[^{14}C]leucine or DL-[^{14}C]-leucine shows a very interesting difference. In Fig. 6 the time course of the reaction is shown for the two forms of [^{14}C]leucine; the total leucine concentration and the specific radioactivity are the same for both curves. It is likely that only the L-isomer is coupled to cell-sap RNA, and inspection of the saturation curves (Fig. 4) shows that the effect of half the concentration of L-isomer in DL-leucine should be a reduction of compound formation by about only 10%, whereas it can be seen in Fig. 6 that on incubation with DL-leucine only 60% of the compound formation occurs as compared with the incubation with L-leucine. That this effect is not just a slowing down through competitive inhibition by the D-isomer of an enzyme system is indicated by the fact that the ratio of compound formation for the two forms of leucine remains the same over the whole time course of the experiment.

It was suggested to us by Dr R. B. Loftfield that the most likely explanation for the excess of labelling in the presence of L-[^{14}C]leucine is the presence of small quantities of other amino acids. This point does, however, need further examination.

The importance of cell-sap RNA in the specific selection of amino acids before their incorporation into the peptide-chain sequence is further emphasized by our experiments with glutamic acid and glutamine. It has been shown by Sansom & Barry (1958) that asparagine and glutamine are incorporated into milk protein independently from aspartic acid and glutamic acid respectively. We were able to show that the binding sites on cell-sap RNA can distinguish between glutamine and glutamic acid. Though the instability of glutamine made it impossible to obtain quantitative information for this amino acid it seems likely that the cell-sap RNA-coupling process is the point in protein synthesis at which the distinction occurs between the dicarboxylic amino acids and their amides.

SUMMARY

1. Systems were developed for the study of the reactions of the enzyme systems and cell-sap ribonucleic acid of guinea-pig mammary tissues with [^{14}C]amino acids and adenosine triphosphate.

2. It was shown that the quantities of amino acids taken up by such systems depend on the amino acid concentration and on the cell-sap ribonucleic acid concentration.

3. Though it is clear that there are specific sites for each amino acid studied, the precise number of sites is difficult to evaluate because of non-specific reactions.

4. From the data obtained the conclusion can be drawn that the extent of combination of amino acids with cell-sap ribonucleic acid determines the quantities of each amino acid to be incorporated into protein. This step may also be responsible for conferring additional specificity on amino acids to determine their addition to polypeptide chains in the correct position in the sequence.

We are grateful to the Rockefeller Foundation for financial support for the purchase of equipment for the Physiology Department, and for the award of a Fellowship to one of us (H.S.). We wish to express our thanks to Dr S. J. Folley, F.R.S., who instigated this programme of research on the biosynthesis of protein in the mammary gland, for his encouragement, to Dr M. Hoagland for much useful advice and to Mrs Margaret Bond for skilful technical assistance.

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Turnover of Nucleic Acids in a Non-Multiplying Animal Cell

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Turnover of nucleic acids may be defined as the continuous renewal of these compounds within the cell by a balanced process of degradation and resynthesis. The idea that ribonucleic acids turn over in this way is derived from experiments on animals (Smellie, 1955). But the complexity of animal experiments does not lend itself to measurement of the rate of intracellular turnover (Cohn, 1957), and the results which have been obtained in such experiments are difficult to interpret. Animal experiments have failed to decide whether turnover of the deoxyribonucleic acids occurs at all. There appear to have been only two studies of nucleic acid turnover in animal cells cultivated *in vitro*. In the first of these (Siminovitch & Graham, 1956) cells in exponential growth were studied. The retention of radioactive phosphorus in the nucleic acids was used to measure turnover, but no turnover was detected either in ribonucleic acid or in deoxyribonucleic acid. In the second study

(Thomson, Paul & Davidson, 1958) the retention of radioactive bases in the nucleic acids was used to measure turnover, and the results appeared to indicate that there was a slow turnover of ribonucleic acid, but no turnover of deoxyribonucleic acid. In this study, however, the growth kinetics of the cell cultures were not measured and repeated subcultures involving traumatic procedures were made during the course of the experiments.

The direct measurement of turnover in nucleic acids entails measurement of the extent to which a radioactively labelled compound which has been incorporated into the nucleic acids is retained in them. However, if turnover occurs, it is possible that the cell may utilize the products of turnover again for the synthesis of new nucleic acids; that is, continuous recycling of these products may take place. If these products are completely utilized, a labelled compound which has been incorporated into the nucleic acids may be completely retained in them. Turnover will not then be detected unless the medium contains some unlabelled compound which has ready access to the cell and which is able to displace or dilute a labelled intermediate in the process of recycling. The nature of the products of nucleic acid turnover, and hence the choice of unlabelled exogenous compounds which might be expected to dilute labelled products, do not appear to have received much attention. In the experiments of Siminovitch & Graham (1956) the nucleic acids were labelled with radioactive phosphorus, and unlabelled orthophosphate was used to dilute any labelled intermediates of turnover which might have been formed; and in the experiments of Thomson *et al.* (1958) the nucleic acids were labelled with radioactive formate, and unlabelled formate was used to dilute the labelled intermediates. But there is no reason to assume that the products of nucleic acid turnover include either orthophosphate or formate; a finding of little or no turnover under these conditions might mean no more than that orthophosphate and formate do not effectively dilute any labelled intermediate.

The experiments described in this paper were carried out on the macrophages of the rabbit. Virtually pure populations of these cells are easily obtained from the peritoneal cavity of the rabbit. The cells remain alive and motile *in vitro* for at least 12 hr., but they do not multiply or grow. They are therefore well suited to studies on turnover, since no corrections need be made for changes in the size of the cell population or in the amount of nucleic acid per cell.

EXPERIMENTAL

Preparation of macrophage cultures. The macrophages were obtained from the peritoneal cavity of the rabbit some days after intraperitoneal injection of liquid paraffin (Lucké, Strumia, Mudd, McCutcheon & Mudd, 1933). The exudates from three rabbits were spun at 220 *g* for 3 min. and the cells were resuspended in 150–200 ml. of medium consisting of 10% of rabbit serum in Hanks's (1948) solution. Measured volumes (10 ml.) of this suspension were introduced into Carrel flasks of 85 mm. diameter. The flasks were incubated at 37° for 40 min. to allow the macrophages to settle and stick to the floor of the flask. The medium was then withdrawn and the adherent layer of cells washed vigorously with physiological saline (0·85% NaCl soln.). Erythrocytes, lymphocytes and dead cells do not adhere to glass, and polymorphonuclear leucocytes adhere poorly. Washing the adherent layer of cells thus removes all but living macrophages and a few cells of fibroblast morphology.

Culture media and radioactive compounds. In the experiments in which the incorporation of radioactive adenine was studied, the cells were maintained in a medium consisting of 25% (v/v) of rabbit serum, which had been dialysed against 0·85% NaCl soln., in Eagle's basal medium (Eagle, Oyama, Levy, Horton & Fleischman, 1956). [8-^{14}C]Adenine was added to make a final concentration of 0·2 mM (0·5–1·0 μc/ml. of medium). In the experiments with radioactive phosphorus the same medium was used, but unlabelled phosphate was omitted. [^{32}P]Orthophosphate was added to make a final concentration of 0·5 μc/ml. of medium. The rabbit serum contained a phosphatase active against adenosine 3′-phosphate and an adenosine deaminase, both of which remained active after dialysis. In experiments involving the use of nucleotides and nucleosides therefore the medium consisted of 20% (v/v) of a 5% (w/v) solution of crystalline human serum albumin in Eagle's medium. The albumin showed no phosphatase or deaminase activity.

When retention of labelled compounds in the nucleic acids was studied the cells were first incubated for 4 hr. at 37° in medium containing the radioactive compound. This medium was then decanted and the cells were washed with a large volume of 0·85% NaCl soln. They were then incubated for 8 hr. in medium containing no radioactive compounds. The various unlabelled purines and purine derivatives were added to this medium at a concentration of 0·2 mM. Eagle's basal medium contains mM-orthophosphate; no extra phosphate was added to this when retention of labelled phosphorus was studied.

Preparation of nucleic acid fractions. The cells were washed three times with 0·85% NaCl soln. and the contents of each flask were harvested in 3 ml. of water containing 1% (v/v) of the detergent Teepol (Shell Chemicals Ltd.); this removed the cells from the glass and completely disrupted them. The solution containing the disrupted cells was made 0·3 N with respect to trichloroacetic acid and the resulting precipitate was spun down; these operations were carried out at 0°. The supernatant was discarded and the precipitate was resuspended in water and spun down again. Lipids were extracted from the precipitate with ethanol at 45° for 30 min. followed by ethanol–ether (1:1) at 45° for 30 min. The residue was hydrolysed with 1 ml. of 0·3 N-KOH for 18 hr. at 37°. 10 N-Perchloric acid was then added at 0° until the pH was 1–2 and the resulting precipitate was spun down at 0°. The supernatant was the ribonucleic acid (RNA) fraction. The precipitate was dissolved in 0·3 N-KOH and reprecipitated by acidification with trichloroacetic acid at 0°; it was then washed with

water and extracted with 0·3 ml. of 0·3 N-trichloroacetic acid at 90° for 30 min. This extract was the deoxyribonucleic acid (DNA) fraction.

Isolation of nucleotides and purine bases. When the phosphorus of the RNA fraction was examined the nucleotides in this fraction were separated by electrophoresis on Whatman no. 1 filter paper in 0·05 M-ammonium formate buffer, pH 3·5; 4 hr. at 13 v/cm. in the apparatus described by Flynn & de Mayo (1951) gave adequate resolution. In other experiments the RNA fraction was hydrolysed with N-HCl at 100° for 1 hr.; this yielded a mixture of purine bases and pyridimidine nucleotides (Loring, 1955). The hydrolysate was evaporated to dryness *in vacuo*. This procedure was altered when it was found that [8-^{14}C]-adenine contributed no radioactivity to the pyrimidine bases. The RNA fraction was then hydrolysed with N-H_2SO_4 instead of N-HCl, and the silver salts of the purines were precipitated (Loring, 1955). The purines were extracted from the silver salts with 0·1 N-HCl and the extract was evaporated to dryness *in vacuo*. The residues were dissolved in 95% ethanol, and applied to sheets of Whatman no. 1 filter paper for chromatography, which was carried out with a descending-solvent system composed of methanol–ethanol–water–conc. HCl (50:25:19:6, by vol.) (Kirby, 1955). The purines of the DNA fraction were also isolated by means of their silver salts and subjected to chromatography in the same system.

Measurements on the culture medium. When the radioactive compounds appearing in the medium were investigated a single flask containing as many cells as would adhere to the floor was used. The cells were incubated in the presence of [8-^{14}C]adenine for 4 hr.; the radioactive medium was removed and the cells were washed once with 0·85% NaCl soln. containing an excess of unlabelled adenine and three times with saline alone. Non-radioactive medium (15 ml.) was then introduced into the flask and incubation continued. Samples (0·5 ml.) of this medium were withdrawn at regular intervals. Each sample of medium was deproteinized with 0·3 N-trichloroacetic acid at 0° and the supernatant was hydrolysed with 0·1 N-H_2SO_4 for 1 hr. at 100°. This hydrolysis gave adequate recoveries. The purines were isolated by precipitation of their silver salts and subjected to chromatography, first in the solvent system described above, which resolved adenine from guanine, and then in the same dimension in butanol–water–ammonia (MacNutt, 1952), which resolved adenine from hypoxanthine. The paper used in these experiments was eluted before use, first with 0·5 N-HCl for 2 days and then with water for 2 days, to remove the ultraviolet-absorbing material which accumulated at the solvent front in the acid system.

The medium remaining at the end of the experiment was deproteinized at 0° with 0·3 N-perchloric acid. After the precipitate had been spun down the supernatant was collected and adjusted to pH 4–5 with 5 N-KOH. The solution was then run through a small column of charcoal (Nuchar C, West Virginia Pulp and Paper Co., N.Y., U.S.A.) and the adsorbed material was eluted with 15 ml. of aq. 40% (v/v) ethanol containing 1% of aq. NH_3 soln. (sp.gr. 0·880) (Cabib, Leloir & Cardini, 1953). The eluate was evaporated to dryness *in vacuo* and subjected to chromatography in the two solvent systems described above.

Determination of specific activity. The radioactive regions on the chromatograms were located by radioautography on X-ray film (Kodak Ltd., Blue Brand). The films were exposed for 3 weeks. Ultraviolet-absorbing regions were located by the method of Holiday & Johnson (1949). These were cut out and eluted overnight into 0·5 ml. of 0·01 N-HCl. The ultraviolet-absorption spectra of the eluates were measured in 1 cm. micro cells in a Hilger Uvispek spectrophotometer. Known volumes of the eluates were dried on glass coverslips and the radioactivity was measured with a mica end-window Geiger–Müller tube (type EHM2S, General Electric Co.).

Radioautographic examination of fixed cell preparations. A monolayer of macrophages was allowed to adhere to a glass slide, and the adherent cells were washed with 0·85% NaCl soln. in the usual way. They were then incubated for 8 hr. at 37° in medium containing tritium-labelled thymidine (Schwarz Laboratories Inc., N.Y., U.S.A.) (0·5 μc/ml. of medium). The specific activity of the thymidine was 360 mc/m-mole. After incubation the preparation was washed with saline and fixed in methanol. The cells were extracted with ice-cold 0·3 N-trichloroacetic acid, washed and dried. Radioautographs were prepared by the stripping-film technique, with Kodak AR. 10 film (Pelc, 1956). The films were exposed for 1 week and then developed. The cells were stained through the film with nuclear-fast red.

Measurement of changes in the ratios of ribonucleic acid and protein to deoxyribonucleic acid. The macrophages were maintained in flasks in medium consisting of 25% of dialysed rabbit serum in Eagle's medium. The cells in one group of six flasks were harvested after incubation for 1 hr. and those in another group of six flasks after incubation for 12 hr. The procedure described for the preparation of nucleic acid fractions was carried out to the stage where the RNA fraction was removed from the precipitate of DNA and protein. The precipitate was dissolved in KOH soln. at pH 12 and the extinction of the solution was measured at 240 and 260 mμ. At 240 mμ the specific extinction of protein is high relative to that of DNA, whereas at 260 mμ the specific extinction of DNA is high relative to that of protein. Change of the ratio of extinctions at these wavelengths therefore provides a measure of any change of the proportion of DNA to protein. Model experiments with mixtures of human serum albumin and purified DNA showed that a 5% change in the proportion of DNA to protein could be detected.

RESULTS

Amount of ribonucleic acid and protein per cell. Over a period of 12 hr. no change in the ratio of RNA to DNA or of protein to DNA could be detected. Since other experiments, which are described below, showed that no synthesis of DNA occurred in the macrophage, it was concluded that the amount of RNA and protein per cell remained constant during the course of the experiments.

Incorporation of [8-^{14}C]*adenine into nucleic acids.* Radioactive adenine, rather than adenosine, was chosen as the precursor of the nucleic acid purines because exogenous adenosine was rapidly deaminated both by the rabbit serum and by the macrophages. Adenine was not deaminated under

the same conditions. When the cells were incubated with [8-^{14}C]adenine, both the adenine and the guanine of the nucleic acids became radioactive. No activity was detected in the protein fraction of the cells.

(*a*) Deoxyribonucleic acid. Macrophages which had been incubated in the presence of tritiated thymidine showed no radioactive labelling in radioautographs. A few cells of fibroblast morphology, which represented less than 1 % of the total cell population, showed heavy nuclear labelling. These cells are known to multiply. Macrophages which had been incubated with [8-^{14}C]adenine were fractionated by the procedure described above for the preparation of nucleic acid fractions. The specific activity of the adenine in the DNA fraction was less than 1 % of the specific activity of the adenine in the RNA fraction. The trace of radioactivity in the adenine of the DNA fraction could be explained by the presence of the few multiplying cells. Since neither adenine nor thymidine was incorporated into the DNA of the macrophages it can be concluded that there was no synthesis of DNA and no turnover of adenine or thymidine residues.

(*b*) Ribonucleic acid. The incorporation of [8-^{14}C]adenine into the purines of the RNA fraction is shown in Fig. 1. The rate of incorporation varied somewhat from experiment to experiment; usually about 20 % of the adenine in the RNA was labelled at the end of 12 hr.

The incorporation of adenine was sensitive to azide: a concentration of 5 mM-sodium azide in the medium caused a 35 % reduction in the amount of labelled adenine incorporated into RNA in 8 hr. The specific activity of the guanine in the RNA was reduced by a similar proportion.

Retention of labelled adenine in ribonucleic acid. When cells which had been labelled by incubation for 4 hr. with [8-^{14}C]adenine were transferred to medium which contained no added purines or purine derivatives none of the labelled adenine was displaced from the RNA during a period of 8 hr. If, however, adenine or certain purine derivatives were added to the medium, labelled adenine was displaced from the RNA (Fig. 2). In the presence of adenine, inosine or the 2′-, 3′- and 5′-phosphate of adenosine, the fall in specific activity was about 20 % in 8 hr. In the presence of inosine 5′-phosphate no fall in the specific activity occurred during a period of 8 hr. Adenosine, however, produced a

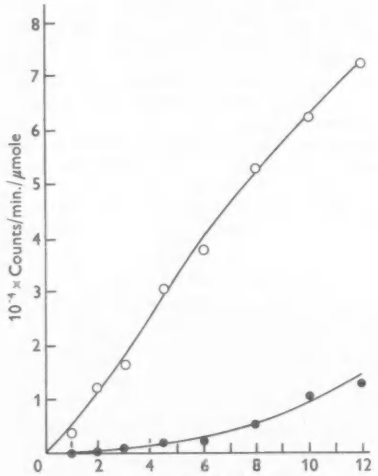

Fig. 1. Incorporation of [8-^{14}C]adenine into the purines of the RNA of macrophages. Macrophages were maintained in medium containing 0·2 mM-[8-^{14}C]adenine for 12 hr. Samples of the cells were harvested at intervals and the specific activities of the adenine and guanine in the RNA were determined. ○, RNA adenine; ●, RNA guanine.

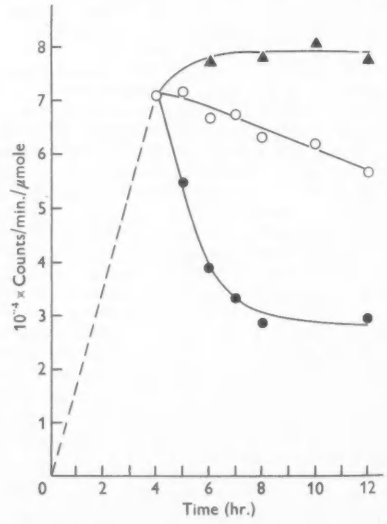

Fig. 2. Retention of radioactive adenine in the RNA of macrophages. The adenine of the RNA was labelled by incubating the cells for 4 hr. in the presence of 0·2 mM-[8-^{14}C]adenine. The cells were then transferred to non-radioactive medium which contained the purine derivative at a concentration of 0·2 mM. Samples of the cells were harvested at intervals and the specific activity of the adenine in the RNA was determined. ▲, Inosine 5′-phosphate in the medium; ○, inosine in the medium; ●, adenosine in the medium.

dramatic effect: in the presence of this compound the specific activity fell by 50 % in about 2 hr. The kinetics of the fall in specific activity in the presence of adenosine are complicated by the fact that the cells rapidly convert exogenous adenosine into inosine. Under the present conditions all the adenosine had disappeared from the medium in about 6 hr. so that during the latter part of the experiment the medium contained mainly inosine, which displaced the labelled adenine from RNA less efficiently than did adenosine.

The fall in the specific activity of the RNA adenine in the presence of exogenous purine and purine derivatives was also azide-sensitive. In the presence of exogenous adenine this fall was about as sensitive to 5 mM-sodium azide as the incorporation of [8-^{14}C]adenine into RNA: the fall in specific activity was reduced by 35 % in 8 hr. In the presence of exogenous adenosine, however, 5 mM-sodium azide reduced the fall in specific activity by only 6–8 % over the same period. This suggests that the incorporation of adenine into RNA involves azide-sensitive processes which are not involved in the incorporation of adenosine.

Appearance of radioactive compounds in the medium. The fall in the specific activity of the RNA adenine was accompanied by the appearance of radioactive compounds in the medium. The increase in the radioactivity of the medium was proportional to the fall in the specific activity of the RNA. However, whatever compound was used to displace the labelled bases from the RNA, the main radioactive compound accumulating in the medium was inosine. Some activity also appeared in a fraction which has been tentatively identified by paper chromatography as guanosine.

The accumulation of radioactive inosine in the medium when labelled cells were incubated in the presence of unlabelled adenosine is shown in Fig. 3. The upper curve shows the specific activity of the inosine fraction; this falls progressively mainly because of the rapid production of unlabelled inosine in the medium by deamination of exogenous adenosine. The lower curve shows the radioactivity

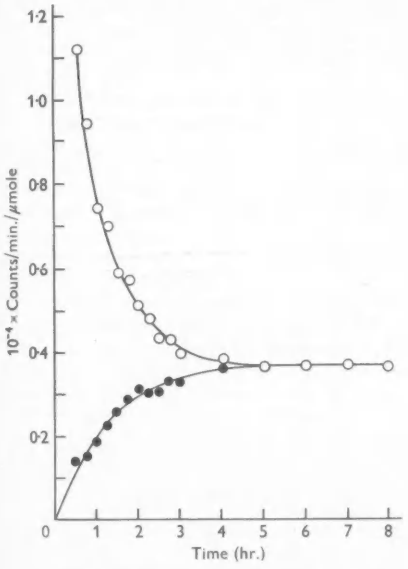

Fig. 3. Accumulation of radioactive inosine in the medium when macrophages in which the RNA had previously been labelled were incubated in non-radioactive medium. The adenine in the RNA had been labelled by incubating the cells for 4 hr. in the presence of [8-^{14}C]adenine. The non-radioactive medium contained 0·4 mM-adenosine. Samples of the medium were taken at intervals and the specific activities of inosine and adenosine were determined. ○, Specific activity of inosine; ●, specific activity of (adenosine + inosine).

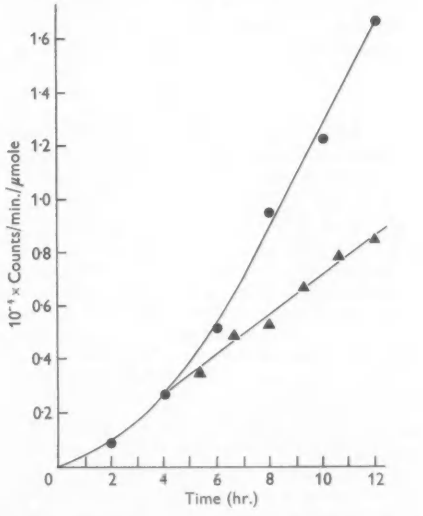

Fig. 4. Incorporation and retention of [^{32}P]phosphate in the cytidylic acid of the RNA. Macrophages were incubated in the presence of [^{32}P]orthophosphate for periods up to 12 hr. Samples were taken at intervals and the specific activity of the cytidylic acid was determined (●). Six flasks were incubated for 4 hr. in the radioactive medium and then transferred to non-radioactive medium containing unlabelled orthophosphate. Samples were again taken at intervals and the specific activity of the cytidylic acid was determined (▲).

in the inosine fraction relative to the total amount of inosine + adenosine present in the medium, thus allowing for the process of deamination. The specific activity of the purines in the medium reached half their maximum value in about 55 min. An apparent equilibrium was reached at the same time as the deamination of exogenous adenosine was complete.

Incorporation and retention of labelled phosphorus in ribonucleic acid. The incorporation of [^{32}P]-phosphate and its retention by the cells were measured under the same conditions as those used to measure the incorporation and retention of [8-^{14}C]adenine. The nucleotides of the RNA fraction were resolved and their specific activities were determined. Fig. 4 illustrates the incorporation and retention of ^{32}P in cytidylic acid; the results obtained with the other nucleotides were similar. It will be seen that the specific activity of the nucleotide continued to rise after the cells had been transferred from the radioactive medium to non-radioactive medium, despite the fact that any residual [^{32}P]orthophosphate was diluted at least 100-fold by the unlabelled orthophosphate of the non-radioactive medium. It thus appears that the labelled intermediates involved in the turnover of RNA are not effectively diluted by unlabelled orthophosphate over the period studied.

DISCUSSION

Since the amount of RNA in the cell remains constant, the incorporation of labelled adenine into RNA and its displacement by certain exogenous purines and purine derivatives indicate that at least some of the RNA is continuously degraded and resynthesized. The half-life of the RNA involved in this turnover is less than 2 hr. The kinetics of the retention of labelled adenine in RNA show that the cell utilizes again the degradation products of RNA turnover with great efficiency (Fig. 2). The observed rate of turnover is therefore determined by the ease with which an unlabelled exogenous compound can enter the pathway by which the degradation products are recycled and so displace or dilute a labelled intermediate. The importance of the choice of the compound to be used for this purpose is shown by the fact that with two of the compounds tested in the present experiments, inosine 5′-phosphate and orthophosphate, no turnover of RNA could be demonstrated over the period studied. With adenine, the 2′-, 3′- and 5′-phosphate of adenosine and inosine, the observed rates of turnover were slower than with adenosine, thus indicating that these compounds had a more restricted access to the turnover pathway than adenosine. But even with adenosine the observed rate of turnover can be regarded only as a minimum value, for it, too, may be limited by the rate at which a labelled intermediate is diluted rather than the rate at which it is recycled. The true rate of turnover remains unknown.

SUMMARY

1. Turnover of ribonucleic acid and deoxyribonucleic acid was studied in rabbit macrophages. These cells remain alive and motile *in vitro* for long periods but do not multiply. The amounts of ribonucleic acid and protein per cell remain constant.

2. No synthesis of deoxyribonucleic acid and no turnover of deoxyribonucleic acid adenine or thymidine residues could be detected.

3. As measured by the incorporation and release of [8-^{14}C]adenine, a rapid turnover of ribonucleic acid occurred. It could be shown, however, that the breakdown products of this turnover were reincorporated into ribonucleic acid with great efficiency.

4. The observed rate of turnover was thus determined by the ease with which an unlabelled exogenous compound could enter the pathway by which the products of turnover were recycled and so displace or dilute a labelled intermediate. In this respect adenine, the 2′-, 3′- and 5′-phosphate of adenosine and inosine were all less effective than adenosine. In the presence of exogenous adenosine the observed half-life of the ribonucleic acid involved in turnover was less than 2 hr.; with the other compounds mentioned the observed half-life was about 12 hr. With inosine 5′-phosphate no turnover could be detected.

5. As labelled adenine was displaced from the ribonucleic acid, a comparable amount of labelled inosine appeared in the culture medium.

6. ^{32}P was incorporated into ribonucleic acid but could not be displaced from it by unlabelled orthophosphate over the period studied.

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A Lipophosphoprotein Complex in Hen Plasma Associated with Yolk Production

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The blood of the laying hen is characterized by a very high lipid content, especially of triglyceride (e.g. Lorenz, Entenman & Chaikoff, 1938; Walker, Taylor & Russell, 1951). It is also characterized by the presence of protein phosphorus ('serum vitellin') demonstrated by Laskowski (1935*a*) and Roepke & Hughes (1935), who observed its virtual absence from the serum of non-laying birds. The high lipid and phosphoprotein content of laying-hen plasma is apparently the result of high oestrogen production (see Lorenz, 1954, and Sturkie, 1954, for reviews).

Laskowski (1935*b*) obtained a phosphoprotein fraction as a precipitate by dilution of laying-hen plasma, and Roepke & Bushnell (1936) observed the precipitation of phosphoprotein on dialysis of hen serum. Both groups of workers, although apparently recognizing the presence of lipid in the precipitable material, made no study of it. More recently, McKinley, Oliver, Maw & Common (1953) and McKinley, Maw, Oliver & Common (1954) presented evidence for the occurrence of phosphoprotein in the material precipitated from the serum of pullets treated with oestrogens, on dilution.

This paper deals with the conditions of preparation of the precipitable lipophosphoprotein from laying-hen plasma, and observations on its composition and properties. In addition its behaviour during the pre- and post-laying periods has been studied. A preliminary account of some of this work has been given (McIndoe, 1957).

METHODS

Animals. Brown Leghorn hens (2 years old), fed on the Poultry Research Centre diet (Bolton, 1958), were used. They were kept in battery cages under artificial lighting. When it was desired to put the hens off lay the amount of light they received was reduced from 14 to 6 hr./day by 1 hr./day (0·5 hr. at either end). To bring them into production this procedure was reversed.

Preparation of precipitable lipophosphoprotein. Blood (usually 3–5 ml.) was taken by syringe from the wing vein and added to a small amount of sodium citrate (about 0·5 mg./ml. of blood). Plasma was diluted with 9 vol. of water, allowed to stand in the refrigerator for 1½–2 hr. and centrifuged at 3000 *g* for 20–30 min. at 2–5°. The supernatant fraction was retained. The precipitate was dissolved in water and reprecipitated by bringing the NaCl concentration to 0·015 M. After standing at 2° for 15 min. the precipitate (PLP) was centrifuged as before for at least 40 min.

Extraction of lipids. Absolute ethanol (20 ml.) was added to the precipitate from 2 ml. of plasma, mixed and allowed to stand overnight. The protein was subsequently extracted with 20 ml. of ethanol–ether (3:1, v/v) three times and finally with ether. The extracts were combined, reduced to less than 10 ml. on a steam bath and evaporated to dryness in a vacuum desiccator over P_2O_5. The supernatant fraction was mixed with 4 vol. of ethanol and the lipids were extracted from the precipitated protein as described above. To remove water-soluble contaminants, water and ether were added to the combined aqueous ethanol–ether extract until an aqueous phase developed. The organic phase was evaporated to dryness as above. Before analysis the dried lipids were dissolved in dry $CHCl_3$.

Analysis of lipids. Total lipid was estimated gravimetrically. (Values for the supernatant fraction are approximate, owing to the relatively low lipid content.) Subsequently the phospholipid ($P \times 25$) was determined by phosphorus analysis (Allen, 1940) and cholesterol by the Liebermann–Burchard reaction (Hawk, Oser & Summerson, 1947). The error in the latter estimation, due to the possible non-equivalent colour intensities of cholesterol and cholesterol esters, is likely to be small since only about 20% of hen-plasma cholesterol is esterified (Walker *et al.* 1951). In a few cases free and total cholesterol were determined by the above-mentioned colorimetric method after using the saponification and digitonin-precipitation procedures of Schoenheimer & Sperry (1934).

Protein. Samples of protein for the determination of N and P were dried at 110° for 18 hr. and allowed to cool in a

desiccator over P_2O_5 before weighing. Nitrogen was estimated by the micro-Kjeldahl method with a copper–selenium catalyst (Chibnall, Rees & Williams, 1943). Total phosphorus was estimated by the method of Allen (1940). Alkali-labile phosphate was determined by incubating the protein in 0·25 N-NaOH soln. at 37° for 24 hr. (Plimmer & Bayliss, 1906), deproteinizing with trichloroacetic acid soln. (5%, w/v) and estimating inorganic phosphate by direct application of the method of Allen (1940); a trace of insoluble material which appeared on addition of molybdate was removed by centrifuging before addition of reducing reagent.

Ultracentrifugal analysis. PLP (0·4 g./100 ml.) in NaCl soln. of sp.gr. 1·006 and in KBr soln. of sp.gr. 1·063 was examined in the analytical ultracentrifuge (Spinco model E) at 52 640 rev./min. and at 18°.

RESULTS

When plasma from the laying hen is diluted with water an abundant precipitate forms almost immediately and this contains a large amount of lipid and also phosphoprotein. It flocculates within about an hour. Formation of a precipitate is not observed with plasma from non-laying birds, except those which will lay within 8 or 9 days or have laid their last egg within 3 days (i.e. birds which have developing ova in their ovary).

Precipitation is apparent at a dilution of 1 in 5 and is maximal at dilutions of 1 in 9 to >1 in 12. The material, after centrifuging, is well packed and has a yellow, translucent, waxy appearance, and can be dissolved in 0·15 M NaCl soln. or in water. It precipitates immediately from aqueous solution on the addition of small amounts of 0·15 M-NaCl. The material then is paler in colour, granular, does not flocculate and packs less well on centrifuging.

PLP, isolated by the procedure given in the Methods section, has a N/P ratio consistently close to 3·5 (Table 1). (All N/P ratios are ratios by weight.) A further three reprecipitations do not significantly alter this ratio, although the solubility of the preparation in water tends to decrease.

Solubility of precipitable lipophosphoprotein. PLP, at a final concentration of 0·2%, was added to NaCl solutions of differing strengths and the amount dissolving estimated by determining N and P. The results (Fig. 1) show that PLP is virtually insoluble in NaCl solutions between 0·01 and 0·04 M and that the N/P ratio is close to 3·5 at all concentrations of NaCl. Prolonged dialysis (18 hr. at 2°) of PLP gives rise to material which also precipitates from dilute NaCl solutions but which has a N/P ratio of about 3, owing to protein (containing no P) remaining in solution.

The solubility of PLP (in 0·2 M-sodium acetate–acetic acid buffer), based on determinations of N and P, is minimal at about pH 5·6 and is almost constant between pH 5·4 and 5·8. The N/P ratio of the precipitated material is near 3·5 at pH 5·0–6·0.

Composition of precipitable lipophosphoprotein. The more detailed analysis of PLP is presented in Table 2, in which results on the plasma concentration are included.

PLP consists of about 20% of protein, containing about 0·75% of P, and 80% of lipid of which 25% is phospholipid and 4% is cholesterol. (Two samples had 76 and 79% of the cholesterol in the free form.) The very high triglyceride content of laying-hen plasma (Walker *et al.* 1951) leads to the conclusion that the remaining PLP lipid is almost entirely triglyceride. The amount of PLP present in the plasma is high and very variable, even in the same hen. It is apparently a function of the number and size of rapidly growing follicles in the ovary (W. M. McIndoe, unpublished work).

After the investigation of PLP began it seemed worthwhile examining the remaining supernatant so that the proportion of whole plasma constituents which are carried by PLP could be assessed. Although the analysis of the supernatant is not entirely satisfactory for phospholipid and phosphoprotein (see Discussion), the results are presented in Table 3, PLP constituents being given as

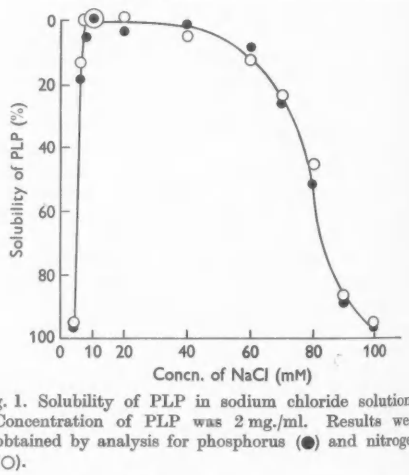

Fig. 1. Solubility of PLP in sodium chloride solutions. Concentration of PLP was 2 mg./ml. Results were obtained by analysis for phosphorus (●) and nitrogen (○).

Table 1. *Precipitable lipophosphoprotein nitrogen and phosphorus content of plasma*

	N (mg./100 ml. of plasma)	P (mg./100 ml. of plasma)	Ratio: N/P*
Mean (10 samples)	60·0	17·2	3·45
Range	49·3–70·0	14·6–20·1	3·26–3·72
S.D.	8·3	2·1	0·13

* Ratio by weight.

Table 2. *Concentration and percentage composition of plasma precipitable lipophosphoprotein*

	PLP (g./100 ml. of plasma)	Protein (N × 7) (% dry wt.)	Protein Ratio: N/P*	Lipid Total (% dry wt.)	Lipid Phospholipid (% total lipid)	Lipid Cholesterol (% total lipid)
Mean (10 samples)	2·42	20·6	19·2	79·4	25·7	4·0
Range	1·36–4·28	19·4–22·5	16·7–22·7	77·5–80·6	24·1–27·3	3·7–4·3
S.D.	0·85	1·0	1·6	1·0	1·0	0·2

* Ratio by weight.

Table 3. *Percentage of whole plasma protein and lipid accounted for by precipitable lipophosphoprotein*

PLP constituents are expressed as percentage of the total in plasma.

	Protein N	Protein P	Lipid Total	Lipid Phospholipid	Lipid Cholesterol
Mean	10·0	36·2	77·7	62·5	67·7
Range	6·4–13·6	22·5–47·8	65·7–92·0	45·1–79·9	49·6–85·3
S.D.	2·7	7·9	7·8	11·0	11·5

a percentage of whole plasma constituents. About 80 % of plasma lipid is associated with PLP, which accounts for smaller proportions of plasma cholesterol and phospholipid. This indicates that almost all the plasma triglyceride is present in PLP. Only about 10 % of plasma-protein N but approximately one-third of the protein P is contained in PLP.

Protein-bound phosphorus. After exhaustive extraction of lipid from PLP with ethanol–ether (3:1, v/v) at room temperature, the protein contains a considerable amount of phosphorus. Four large-scale preparations of PLP protein having a nitrogen content of 14·3, 14·5, 14·1 and 14·3 % contained 0·79, 0·74, 0·71 and 0·72 % of P respectively. (N/P ratios on a larger group are included in Table 2.) Most of this P is labile to alkali (0·25 N-NaOH at 37° for 16 hr.), 80–85 % being liberated as inorganic phosphate and only about 2 % remaining acid-insoluble. No more phosphate is released on further incubation (up to 60 hr.) and the liberation is virtually complete in 10 hr.

Similar results are obtained with the lipid-extracted protein in the supernatant obtained on precipitation of PLP. However, the proportion of protein P which is labile to alkali is lower and more variable (53, 50, 61 and 67 % for four samples) and the proportion remaining acid-insoluble (6 %) is greater than for PLP protein.

Stability of precipitable lipophosphoprotein to organic solvents. Examination of the stability of PLP lipid to organic solvents has not been extensive. Only trace amounts of lipid are immediately extracted from a PLP solution by gentle rotation (without breaking the interphase) with an equal volume of ether. About 50 % of the lipid (30 % of the phospholipid) passes into the ether phase on standing for 4 hr. at 20°, although a latent period of somewhat less than 1 hr. is apparent before appreciable amounts are extracted. Approximately 75 % (including 50 % of the phospholipid) is removed if the ether contains 10 % (v/v) of ethanol (see Macheboeuf & Sandor, 1932; Macheboeuf, 1953), and the latent period is probably reduced. If the PLP solution is vigorously shaken with ether almost 85 % of the lipid (60 % of the phospholipid) is extracted much more rapidly, though a short latent period is again observed. Similar results are obtained if the PLP solution is frozen below −25° in the presence of ether (McFarlane, 1942).

Heterogeneity of precipitable lipophosphoprotein. After most of the work presented was completed it became possible to examine PLP in an analytical ultracentrifuge. In a NaCl soln. of sp.gr. 1·006 two components separate, one of which floats (about 70 % of the total calculating from the schlieren diagram: apparent S value −11) and one sediments (apparent S value 13). A qualitatively similar result is obtained in a potassium bromide solution of sp.gr. 1·063.

Precipitable lipophosphoprotein during pre- and post-ovulatory periods. PLP appears in the plasma of hens 7–8 days before the first ovulation (after they have been out of lay for at least 3 weeks) and increases in amount, reaching a maximum about 3 days before the first ovulation. Thereafter the plasma concentration decreases, reaching the relatively constant values for highly productive hens 2–5 days after the first ovulation (see Fig. 2 for a characteristic pattern). The composition of PLP changes slightly during this period (Table 4). The

initially higher protein and lower lipid content quickly revert to characteristic values as the concentration of PLP increases and the relatively high cholesterol content drops progressively.

Analysis of plasma before PLP is detectable and subsequently of the supernatant from the precipitation of PLP shows (Fig. 2) that the protein-bound P behaves similarly to PLP, although it appears in the plasma at least 24 hr. before the latter is evident. The lipids in the supernatant show a less marked but earlier increase, and the maximum occurs on the sixth or seventh day before ovulation. Supernatant protein N varies little during the pre-ovulatory period, though more often than not it is higher at the beginning than when egg-laying is established and drops gradually throughout the period.

The investigation of PLP during the cessation of egg production has been less extensive. However, it is clear from Table 5 that PLP begins to disappear about the day of the last ovulation and is not present 4 days later. Instead, only a small amount of protein, containing trace amounts of lipid, is precipitable on dilution. This protein contains no P. In some individual birds this protein may persist for at least 14 days but disappears within 3 weeks. Coincident with the disappearance of PLP, protein P drops sharply almost to zero in the supernatant fraction of the plasma, supernatant total lipid and phospholipid decrease slightly and cholesterol increases from approximately 30 mg./100 ml. of plasma, before PLP decreases, to about 100 mg./100 ml. of plasma, when PLP has disappeared. The latter value is slightly lower than that for highly productive hens.

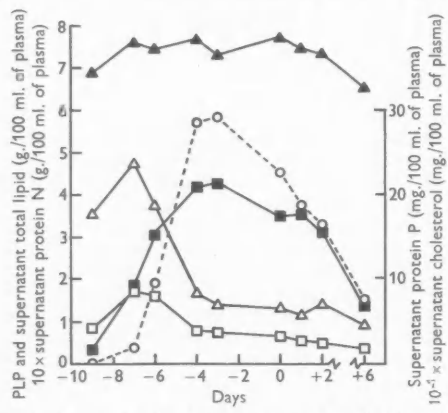
PLP and supernatant total lipid (g./100 ml. of plasma)
10 × supernatant protein N (g./100 ml. of plasma)
Supernatant protein P (mg./100 ml. of plasma)
10⁻¹ × supernatant cholesterol (mg./100 ml. of plasma)
Days
−10 −8 −6 −4 −2 0 +2 +6
0 1 2 3 4 5 6 7 8
10 20 30

Fig. 2. Changes in the plasma concentration of PLP and of protein and lipid constituents of the supernatant (after removal of PLP) during the period when hens are coming into lay. Results are for one bird characteristic of a group of six. Abscissa gives time of sampling in days before (−) and after (+) the first ovulation (0). ○, PLP. Supernatant constituents: ▲, protein nitrogen; ■, protein phosphorus; △, cholesterol; □, total lipid; (phospholipid follows a pattern similar to that for total lipid but at a slightly lower level).

DISCUSSION

The constancy of composition of PLP prepared from different plasma samples and the results obtained on PLP precipitated at different sodium chloride concentrations and by isoelectric precipitation do not suggest that PLP is heterogeneous. However, examination in the analytical ultracentrifuge shows PLP to consist of two components, one of which is a light lipoprotein and the other a protein or dense lipoprotein. This fact should be kept in mind throughout the following discussion.

PLP contains a very high proportion of lipid (80%). The apparent triglyceride content is very high and the cholesterol content is relatively low. Indeed the gross composition of the lipid is very similar to that of whole yolk. Also, it accounts for about 80% of whole plasma lipid and apparently an even greater proportion of the plasma triglyceride. These facts, together with its behaviour when hens begin or stop laying, lead to the conclusion that PLP is carrying a very high proportion of the plasma lipid destined for inclusion in the

Table 4. *Composition of precipitable lipophosphoprotein during the period when hens are coming into lay*

Samples, taken from the same bird, are designated days before (−) or after (+) the first ovulation (0) and are those given in Fig. 2.

	Protein		Lipid		
Days	(N × 7) (% dry wt.)	Ratio: N/P*	Total (% dry wt.)	Phospholipid (% total lipid)	Cholesterol (% total lipid)
−7	30·5	28·8	69·5	28·9	11·5
−6	21·2	23·8	78·8	21·5	10·6
−4	17·2	19·3	82·8	20·6	8·0
−3	17·6	18·8	82·4	21·6	7·8
0	18·1	20·0	81·9	21·0	6·7
+1	18·7	19·2	81·3	22·9	6·5
+2	19·1	19·4	80·9	23·1	6·5

* Ratio by weight.

Table 5. *Composition and concentration of precipitable lipophosphoprotein when hens are going off lay*

Samples are designated days before (+) or after (−) the last ovulation (0).

		Precipitated material		Composition of precipitated material					
				Protein			Lipid		
Bird no.	Days	g./100 ml. of plasma	Ratio: N/P*	% dry wt. (N × 7)	mg. of N/ 100 ml. of plasma	Ratio: N/P*	Total (% dry wt.)	Phospholipid (% dry wt.)	Cholesterol (% dry wt.)
5	+5	1·63	3·82	19·6	45·6	24·9	80·4	22·5	3·4
4	+3	2·20	5·31	20·0	62·9	27·9	80·0	15·0	3·9
10	+3	1·87	5·70	21·4	57·2	20·0	78·6	13·2	2·1
11	+3	0·87	3·28	21·8	57·1	25·3	78·2	31·3	3·2
1	0	1·31	3·52	20·6	38·6	25·2	79·4	26·4	3·0
3	−2	0·71	4·62	23·8	24·1	27·0	76·2	23·7	3·1
1	−3	0·21	22·5	50·0	16·2	50·6	50·0	5·0	—§
3	−4	<0·10	44·3	90·0	8·8	—‡	10·0	5·0	—
8	−4	<0·10	—†	>90·0	12·0	—	Trace	<1·0	—
6	−5	<0·10	—	>90·0	10·2	—	Trace	<1·0	—
7	−5	<0·10	—	>90·0	4·4	—	Trace	<1·0	—
9	−5	<0·10	—	>90·0	9·4	—	Trace	<1·0	—
12	−5	<0·10	—	>90·0	11·3	—	Trace	<1·0	—
2	−7	<0·10	—	>90·0	10·0	—	Trace	<1·0	—

* Ratio by weight.
† Only a trace of phospholipid phosphorus was present.
‡ Phosphorus not detectable.
§ Not estimated.

yolk. Recently a fraction with properties similar to those of PLP has been isolated from yolk (W. M. McIndoe, unpublished work) and is being investigated. However, according to Schmidt, Bessman, Hickey & Thannhauser (1956), only a small proportion of yolk triglyceride appears to be associated with phosphoprotein.

Recently, electrophoresis and the ultracentrifuge have been used to examine the plasma lipoproteins of the domestic fowl. Electrophoresis has demonstrated the occurrence of a lipoprotein component in the serum of the laying hen which is not obvious in immature birds (Moore, 1945, 1948). It apparently contains a high proportion of lipid but the protein accounts for only a small proportion of total serum protein. PLP may be related to this lipoprotein. There is also an increase in lipoprotein of laying hens in the β-globulin region and a decrease in the α_1-globulin area (Moore, 1948). This picture has been confirmed in general by Brandt, Clegg & Andrews (1951) and Clegg & Hein (1953) in hens and by Clegg *et al.* (1951) and Clegg & Hein (1953) in cocks treated with oestrogens. The paper-electrophoresis study of serum from laying hens and pullets treated with oestrogens, by McKinley *et al.* (1953, 1954), also confirms these findings in part.

Schjeide (1954), using the ultracentrifuge, has observed the occurrence of a large amount of lipoprotein in laying-hen serum corresponding to β-lipoprotein and lighter components. Hillyard, Entenman & Chaikoff (1956), using the same technique, have examined the serum of cocks treated with oestrogens and find large amounts of a fraction having a high triglyceride content and floating at a lower specific gravity than does β-lipoprotein. It is not present in normal cock serum. This fraction is probably related to the low-density component of PLP. These workers also observed an increased β-lipoprotein fraction and a decreased α_1-lipoprotein fraction in the serum of cocks treated with oestrogens. The report of Schjeide & Urist (1956) indicates a similar situation.

The lipid composition of the plasma of laying hens compared with that of non-laying birds is a reflexion of the changes in the lipoprotein fractions. The decrease in α_1-lipoprotein, which is relatively rich in cholesterol and especially phospholipid (Hillyard *et al.* 1956), would tend to offset the effect of the appearance of PLP and the increase in the β-lipoprotein fraction, at least for cholesterol and phospholipid. (Note the similar plasma cholesterol of layers and non-layers.)

The protein moiety of PLP, containing about 0·74% of phosphorus, is similar to that which Laskowski (1935*b*) prepared from the original precipitate obtained by ten times dilution of serum at pH 5·0–5·5 and which contained 0·53% of phosphorus (the lower pH may have caused precipitation of material in addition to PLP). By salt-fractionation of the original precipitate Laskowski (1935*b*) obtained a component with a protein phosphorus content of 0·9%. A fraction similar to the latter was obtained by Roepke & Bushnell (1936) from material precipitated from laying-hen serum on dialysis. In agreement with the latter workers the material precipitated by dilution contains less than half the total plasma-protein phosphorus.

The present methods of estimating phosphoprotein phosphorus are not satisfactory (Ågren, de Verdier & Glomset, 1954). Methods based on the lability to alkali of phosphoprotein phosphorus (Plimmer & Bayliss, 1906) may give low results owing to incomplete liberation of phosphate; phosphoserine and phosphoserine peptides have been detected in alkaline hydrolysates of proteins (Hutchison *et al.* 1956; Phillips, 1956; Glomset, 1957; Hawthorne, 1957). Diesterified protein phosphate may be more stable than the monoester form. Also the amino acid environment of the phosphoseryl residues may influence the proportion of inorganic phosphate liberated, as is indicated by the work of Plimmer & Bayliss (1906) and of Rimington (1941).

In the present work it has been presumed that all the protein-bound phosphorus is phosphoprotein phosphorus. This may be true of PLP but the protein of the plasma supernatant fraction is probably contaminated with phospholipid in a form non-extractable by ethanol (McIndoe, 1958). Thus in this fraction estimates of phosphoprotein phosphorus may be high and phospholipid estimates correspondingly low. The protein phosphorus of PLP behaves towards alkali in a way very similar to that found by Plimmer & Scott (1908) for 'vitellin' phosphorus. It should be pointed out that no phosphorylated amino acid has been isolated from the proteins of laying-hen plasma, though phosphoserine has been isolated from yolk proteins (Lipmann & Levene, 1932; Levene & Schormuller, 1933).

In the hen follicles begin to grow rapidly 7–9 days before ovulation (Romanoff, 1931, 1943; Warren & Conrad, 1939; Entenman, Ruben, Pearlman, Lorenz & Chaikoff, 1938; Lorenz, Pearlman & Chaikoff, 1943), and the appearance of PLP 7 or 8 days before ovulation coincides with this. Also, PLP and phosphoprotein disappear rapidly from the plasma when egg production ceases, in agreement with Hosoda, Kaneko, Mogi & Abe (1955), who have shown the disappearance of 'serum vitellin' by immunological methods within 5 days of the last egg being laid.

The total protein content of the plasma is apparently similar in laying and non-laying hens, though the range of values is large (D. J. Bell, unpublished work; Sturkie & Newman, 1951; see also Sturkie, 1954). However, during a short immediately pre-ovulatory period the plasma protein level is higher than it is in the truly non-laying state or when egg-laying is established. This is due to the presence of a large amount of PLP at this time (Fig. 2). This is in confirmation of Greenberg, Larson, Pearson & Burmester (1936) and of Vanstone, Maw & Common (1955), who worked with fowls, and of McDonald & Riddle (1945), working with pigeons. Other workers have made similar observations on the high pre-ovulatory level of lipids in the plasma of doves and pigeons (Riddle & Burns, 1927; Riddle, 1942), and in the blood of hens (Lorenz *et al.* 1938; Chaikoff, Lorenz & Entenman, 1941), and of protein phosphorus in pigeon plasma (McDonald & Riddle, 1945). These high values may be partially attributed to the submaximal size of the growing follicles which are 'absorbing' material into the yolk. However, on the day of the first ovulation PLP and phosphoprotein are still higher than when high egg production is established. The very high protein phosphorus content of the supernatant (from PLP preparation) during the pre-ovulatory period without an increase in protein nitrogen may be explained if, as in egg yolk, most of the phosphoprotein phosphorus is present as phosvitin (Mecham & Olcott, 1949), which contains 10 % of phosphorus.

Changes in the composition of PLP occur when hens are coming into and going out of production, i.e. at times when their physiological state is altering rapidly. This may be due to an alteration in the proportions of the components of PLP separable in the ultracentrifuge or in the composition of these components. The effect of nutritional or hormonal changes in altering lipoprotein composition has been noted by Macheboeuf (1929), Macheboeuf & Rebeyrotte (1949) and by Hillyard *et al.* (1956).

PLP should provide very suitable material for the investigation of the chemistry and biosynthesis of lipoprotein (of high triglyceride content) and phosphoprotein. It should also supply the means of elucidating the problem of yolk formation, i.e. the selective 'absorption' of high-molecular-weight substances by the hen ovary against a concentration gradient.

SUMMARY

1. A lipophosphoprotein complex (PLP) of constant composition can be precipitated from laying-hen plasma by dilution with water, and a method for its preparation and estimation has been developed. It is not detectable in the plasma of non-laying hens or of cocks.

2. The concentration of PLP is about 2 g./100 ml. of plasma and is very variable. It consists of about 20 % of protein and 80 % of lipid (of which 25 % is phospholipid and 4 % is cholesterol, largely free). The remaining lipid is probably largely or wholly triglyceride. The protein contains approximately 0·75 % of phosphorus (80 % of which is very labile to alkali, indicating that it is phosphoprotein).

3. PLP accounts for a large but variable proportion of whole plasma lipids; i.e. total lipid, about 80 %; phospholipid and cholesterol, approxi-

mately 65 %. It also accounts for about 35 % of the plasma protein phosphorus and 10 % of the protein nitrogen.

4. PLP appears in hen plasma about 7 days before the first ovulation and disappears within a few days when hens go off lay. The plasma concentration and composition of PLP during these periods have been examined. Details of changes in the lipid and protein nitrogen and phosphorus of the supernatant obtained from the preparation of PLP are also given.

5. Preliminary ultracentrifugal analysis of PLP has shown it to consist of two components. The major one floats in a sodium chloride solution of specific gravity 1·006, with an apparent $S -11$, and the other sediments (apparent S 13).

6. It is possible that PLP transports most of the plasma lipid and almost half the plasma phosphoprotein included in egg yolk.

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Studies on the Conversion of Glucose into Lactic Acid in the Ehrlich Ascites Tumour

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The most widely substantiated biochemical characteristic of neoplastic tissue is the high rate of conversion of glucose into lactic acid in the presence of oxygen, first described by Warburg (1930). The significance of this high aerobic glycolysis remains controversial (Warburg, 1956; Weinhouse, 1956; Burk, 1956). Another characteristic of neoplastic tissue is the depression of the respiration rate on adding glucose, described by Crabtree (1929). This Crabtree effect has recently been confirmed and studied by many others (Brin & McKee, 1956; Chance, 1953; Chance & Hess, 1956; Gatt, Krimsky & Racker, 1956; Ibsen, McCarty & McKee, 1956; Kun, Talalay & Williams-Ashman, 1951; McKee, Lonberg-Holm & Jehl, 1953; Bloch-Frankenthal & Weinhouse, 1957; Emmelot, 1958).

The present study was initiated from the point of view of radiotherapy. Oxygen is known to be an important factor in the sensitivity of biological material to ionizing radiations (Crabtree & Cramer, 1934). It has been suggested that the oxygen tension in some neoplastic tissue may be sufficiently low to affect adversely the benefit available from radiotherapy (Thomlinson & Gray, 1955). This has, in fact, been shown in model systems (Scott, 1957), and more recent clinical trials of oxygen administered under pressure during irradiation have given promising results (Churchill-Davidson, Sanger & Thomlinson, 1957).

The inhibition of respiration of neoplastic cells by glucose seemed to be an attractive method of decreasing tissue-oxygen gradients, and thereby preferentially increasing the oxygen tension inside a tumour, while having little or no effect on the blood supply or on the oxygen tension of neighbouring healthy tissue. Before studying the oxygen at different concentrations of blood glucose *in vivo*, more information was required on the nature of the effect of glucose and of the conditions for maximum inhibition of respiration *in vitro*. Ehrlich ascites tumour cells were used for these studies, since their rate of respiration is very dependent on glucose concentration.

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The glucose and lactic acid biochemistry of Ehrlich ascites tumour cells in mice has already been studied in considerable detail. Glucose is obtained from the blood supply of the host via the peritoneal fluid at sufficient rate to maintain the cells in maximum glycolysis, the lactic acid produced being returned to the blood (Kemp & Mendel, 1957). *In vitro* the cells respire rapidly for many hours without added glucose. Their respiratory quotient indicates that they are not metabolizing glucose (Dickens & Simer, 1930), but probably some lipid substance (Slechta, Jakubovic & Sorm, 1955). When glucose is added the rate of respiration falls, and the glucose is rapidly converted into L-lactic acid (Brin, 1953). The carbon dioxide produced comes partly from the residual respiration and partly from oxidation of some of the glucose (Bloch-Frankenthal & Weinhouse, 1957). No single theory to explain the effect of glucose on respiration appears to have received general acceptance, and more experimental evidence seemed desirable.

MATERIALS AND METHODS

The Ehrlich ascites tumour cells used in this study were peritoneal transplants of 5–8 days in mice of a heterogeneous strain. The mice were killed and the fluid was withdrawn immediately before each experiment. The fluid was pooled from the minimum number of mice required to provide sufficient material. In order to approximate as nearly as possible to the conditions *in vivo*, the ascitic fluid was used as such without further treatment, and always contained some blood. The Ehrlich ascites tumour was the tetraploid strain of Klein. Ascitic fluid from a tumour-bearing mouse contained a remarkably constant number of cells, $1{\cdot}309 \times 10^8 \pm 0{\cdot}043 \times 10^8$/cm.^{3}, with an average cell volume of 1903 μ^3 and variation coefficient of 15 % (Klein & Revesz, 1953). Each experiment consisted of a comparison between control and experimental portions taken from the same pooled fluid. Cell counts were not always made; thus no comparison should be made between the absolute values obtained in different experiments.

Measurements of respiration were made with the conventional Warburg technique. The main compartment of the manometer flasks contained 1·0 ml. of buffer, and either 1·0 ml. of ascitic fluid or 0·5 ml. of ascitic fluid, and 0·5 ml. of 0·154 M-NaCl soln. Glucose in 0·5 ml.

of 0·154 M-NaCl soln. was added by tipping in from the side bulb; 0·5 ml. of 0·154 M-NaCl soln. containing no glucose was always tipped into control flasks at the same time. Carbon dioxide was absorbed on filter paper saturated with 0·15 ml. of 5 N-KOH soln. in the centre well. Measurements were made at 37° in an atmosphere of air. The buffer at pH 7·4 was made by mixing 4·3 vol. of 0·154 M-Na_2HPO_4 to 1 vol. of 0·154 M-NaH_2PO_4. The buffer at pH 6·0 was made by mixing 1 vol. of 0·154 M-Na_2HPO_4 with 7·3 vol. of 0·154 M-NaH_2PO_4. Potassium salts were avoided after the inhibitory action of high concentrations of K^+ ions (McKee *et al.* 1953) had been confirmed in this Laboratory. The buffer concentration had to be kept as high as was possible without upsetting the osmotic pressure of the system, to avoid gross changes in pH during the experiment, from the large quantities of lactic acid produced. Even so, the pH at the end of an experiment was frequently as much as 0·2 pH unit lower than at the beginning.

Glucose measurements were made by the method of King (1951). A sample (0·1 ml.) of the fluid was added to 1·8 ml. of isotonic $CuSO_4$ reagent (made by adding 6·06 g. of anhydrous Na_2SO_4 and 3·0 g. of $CuSO_4,5H_2O$ to 1 l. of water). Aqueous 10% (w/v) $Na_2WO_4,2H_2O$ soln. (0·1 ml.) was added and the mixture shaken and centrifuged at 540 *g* for 5 min. Glucose was estimated in a portion of the supernatant fluid. Lactic acid was measured by the method of Barker & Summerson (1941); 0·5 or 1·0 ml. of 10% (w/v) trichloroacetic acid in water was added to the 0·5 or 1·0 ml. sample to be estimated respectively. The mixture was diluted with water to 25 ml. and centrifuged at 970 *g* for 5 min., and lactic acid determined on portions of the supernatant.

The volume of the liquid trapped between centrifuged cells was measured by suspending about 10 ml. of cells in 100 ml. of sucrose–NaCl soln. (containing 5 g. of sucrose and 8·15 g. of NaCl/l.), centrifuging for 10 min. at 940 *g* and resuspending in fresh sucrose–NaCl soln. A portion was then centrifuged under standard conditions (970 *g* at the tip of the centrifuge tube for 5 min.) in a graduated tube. The total volume of the packed cell mass was noted. The supernatant was poured off and replaced with a volume of NaCl soln. exactly equal to the volume of the cell mass and mixed by vigorous shaking. A sample (1 ml.) was diluted to 50 ml. with 0·154 M-NaCl soln., the cells were removed by centrifuging for 10 min. at 940 *g* and sucrose was determined in a sample of the supernatant by the anthrone method of Seifter, Dayton, Novic & Muntwyler (1950). The mean of eight determinations showed that 1 ml. of cells centrifuged under these standard conditions contained 0·44 ± 0·04 ml. of fluid between them.

RESULTS

Fig. 1 is a typical curve obtained by plotting the rate of respiration with and without added glucose against time. In this particular experiment no buffer has been added. Even so, the respiration continues at a high rate for many hours although falling slightly with time. The rate of respiration is significantly less in the presence of glucose.

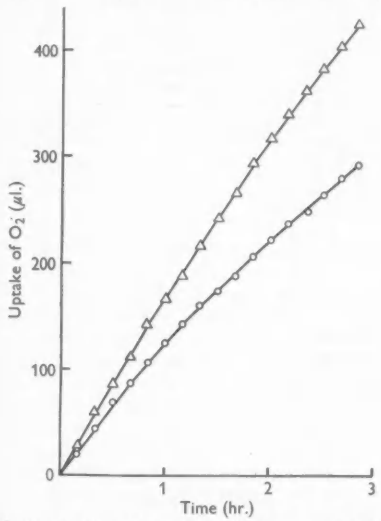

Fig. 1. Respiration of Ehrlich ascites tumour cells at 37° in air in Warburg manometer; CO_2 was removed by KOH. △, 1 ml. of cells + 1·5 ml. of 0·154 M-NaCl soln.; ○, 1 ml. of cells + 1·5 ml. of 0·154 M-NaCl and glucose soln. to a final concentration of 400 mg./100 ml.

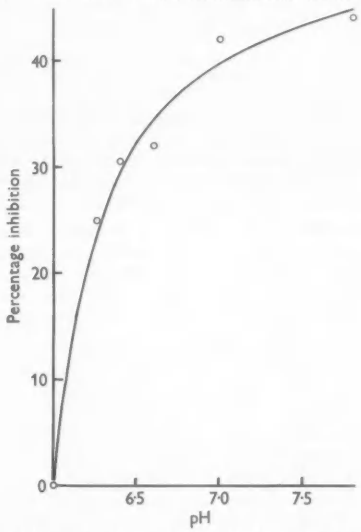

Fig. 2. Effect of pH on the magnitude of inhibition of respiration rate of Ehrlich ascites tumour cells on adding glucose. Cell suspension (1·0 ml.) and 1·0 ml. of 0·154 M-phosphate buffer at the appropriate pH were added to each Warburg flask. The figures are the fall in respiration rate on tipping in glucose in 0·154 M-NaCl soln. to a final concentration of 1000 mg./100 ml., as percentages of the rate in the control flask in which 0·154 M-NaCl soln. only was tipped.

Fig. 2 shows the relationship between the extent of the inhibition of respiration (Crabtree effect) in Ehrlich ascites tumour cells and the pH of the buffer added. The figures for the percentage inhibition are the respiration rates in the absence of glucose, minus the respiration rates in the presence of glucose, as a percentage of the respiration rates in the absence of glucose, the rate of respiration being determined from the initial slope of the line obtained by plotting uptake against time. The Crabtree effect virtually disappears at pH 6·0. Fig. 3 shows that this is not due to an inability of the cells to utilize glucose at this pH. Although the rate of consumption of glucose is less at pH 6·0 than at pH 7·4, it is still far from negligible.

Since the rate of utilization of glucose by Ehrlich ascites tumour cells is very high, care is required to ensure that sufficient glucose remains during the entire experiment to avoid spurious results. Figs. 4 and 5 show the effect of adding high and low concentrations of glucose to these cells. The result of using adequately high initial concentrations of glucose (above 300 mg./100 ml.; Fig. 4) is that it is not completely consumed during the experiment, and the concentration of the lactic acid produced continues to rise. In Fig. 5 the smaller amount of glucose added is quickly consumed, and the lactic acid concentration ceases to rise and eventually starts to fall.

In Fig. 6 the initial change in respiration rate on adding glucose is plotted against the glucose concentration. The Crabtree effect is nearly independent of the glucose concentration above 100 mg./100 ml., but begins to fall off more rapidly below this level. Thus it was necessary to ensure that the glucose concentration remained above 100 mg./100 ml. throughout the experiment. For this

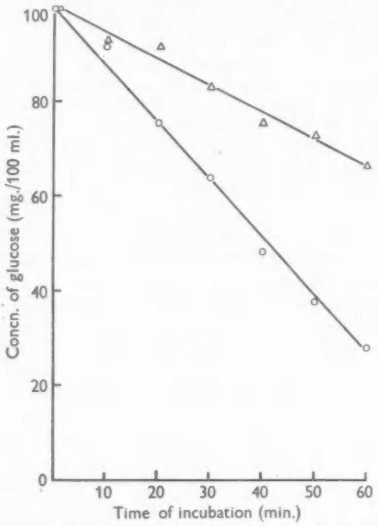

Fig. 3. Rate of removal of glucose from Ehrlich ascites tumour cells shaken in air at 37°. The system contained cell suspension (2 ml.) and 2 ml. of 0·154 M-NaCl soln. and 4 ml. of buffer, pH 6·0 (△) or pH 7·4 (○), and glucose to a final concentration of 100 mg./100 ml.

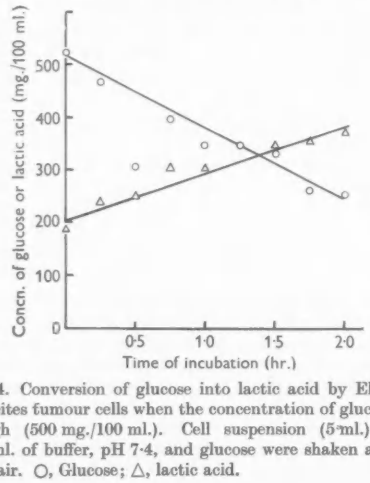

Fig. 4. Conversion of glucose into lactic acid by Ehrlich ascites tumour cells when the concentration of glucose is high (500 mg./100 ml.). Cell suspension (5 ml.) and 5 ml. of buffer, pH 7·4, and glucose were shaken at 37° in air. ○, Glucose; △, lactic acid.

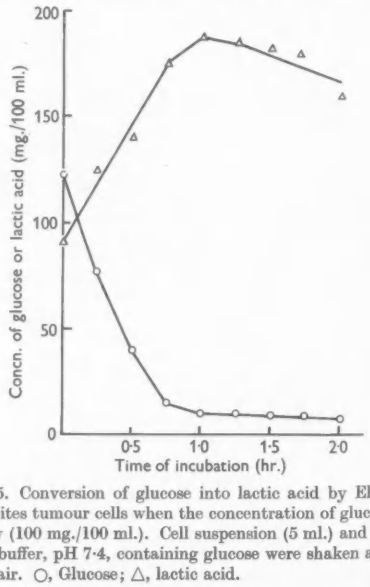

Fig. 5. Conversion of glucose into lactic acid by Ehrlich ascites tumour cells when the concentration of glucose is low (100 mg./100 ml.). Cell suspension (5 ml.) and 5 ml. of buffer, pH 7·4, containing glucose were shaken at 37° in air. ○, Glucose; △, lactic acid.

Table 1. *Distribution of lactic acid between Ehrlich ascites tumour cells and the peritoneal fluid*

Control cells were centrifuged immediately after withdrawal. Glucose in 0·154 M-NaCl soln. was added to treated cells to a final concentration of 90·9 mg./100 ml. and shaken in air at 37° for 10 min., then cooled in ice and centrifuged. All readings are averages of duplicate samples.

	Lactic acid (mg./ml. of sample)		
	Centrifuged supernatant	Centrifuged cell fraction	Cells corrected for trapped supernatant
Control (no glucose)	0·78	0·31	0·00
Glucose added	1·06	0·86	0·70

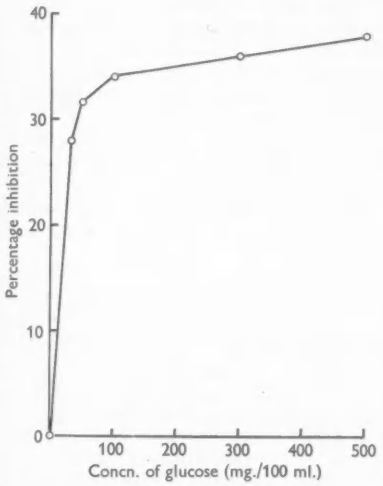

Fig. 6. Effect of glucose concentration on the inhibition of the respiration rate of Ehrlich ascites tumour cells. The system contained cell suspension (1 ml.) and 1 ml. of 0·154 M-buffer, pH 7·4, and the requisite glucose in 0·5 ml. of 0·154 M-NaCl soln.

reason, 1000 mg. of glucose/100 ml. was used as the starting concentration in Fig. 2.

To test whether the resultant lactic acid was in part responsible for the Crabtree effect, an analysis for lactic acid was carried out on the cells and on the fluid surrounding them. In a typical experiment, a sample (6·9 ml.) of ascitic fluid containing Ehrlich ascites tumour cells was immediately cooled in ice–water and, within 10 min. of collection, was centrifuged for 5 min. at 970 *g*. The supernatant was carefully removed by pouring into a separate tube and drying the wet glass surface with filter paper, leaving 3·05 ml. of hard-packed cells. 0·154 M-Sodium chloride soln. (3·05 ml.) was added and thoroughly mixed. The dilution was necessary to make the cell suspension sufficiently fluid to be accurately pipetted. Tri-

Table 2. *Distribution of lactic acid between washed Ehrlich ascites tumour cells and the peritoneal fluid*

Cells were first washed almost free of lactic acid by centrifuging and resuspending in 0·154 M-NaCl soln. Glucose in 0·154 M-NaCl soln. was added to a final concentration of 90·9 mg./100 ml. Cells were shaken in air at 37° and samples withdrawn at intervals. All readings are averages of duplicate samples.

	Lactic acid (mg./ml. of sample)		
Time (min.)	Centrifuged supernatant	Centrifuged cell fraction	Cells corrected for trapped supernatant
10	0·57	0·67	0·75
210	0·64	0·58	0·53
315	0·52	0·42	0·34

chloroacetic acid was added to duplicate 0·5 ml. samples of supernatant and duplicate 1·0 ml. samples of the diluted cell suspension. All samples were made up to 25 ml. with water for lactic acid analysis.

The results given in Table 1 show that when a correction was made for the supernatant fluid mechanically trapped between the centrifuged cells (see Methods) there was virtually no lactic acid inside the cell. This represents the state of ascites cells *in vivo* where the glucose concentration is known to be extremely low (Kemp & Mendel, 1957). In a similar experiment made in the presence of glucose, 0·5 ml. of 0·154 M-sodium chloride solution containing 5 mg. of glucose was added to 5 ml. of freshly drawn tumour bearing ascitic fluid. The mixture was shaken in a conical flask in an adapted Warburg water tank at 37° in air for 10 min. and then cooled in ice–water. The cells were centrifuged and lactic acid was estimated as before. The lactic acid concentration in the cells, although still less than in the supernatant, had risen to a relatively high value (Table 1). The concentration in the supernatant is greater than in the previous experiment, being the sum of the lactic acid originally present and that amount released by the cells fermenting glucose.

In the next experiment (Table 2) the cells in 19 ml. of tumour-bearing ascitic fluid were first washed almost free of lactic acid by centrifuging at 970 *g* for 5 min. and resuspending them in 0·154 M-sodium chloride solution to a final volume of 19 ml. 0·154 M-Sodium chloride solution (1·9 ml.) containing 19 mg. of glucose was added and the mixture was shaken in a stoppered conical flask containing air in the water bath at 37°. Samples (5 ml.) were withdrawn at intervals, cooled in ice–water, centrifuged and lactic acid determinations made on cells and supernatant as before. In this experiment, after 10 min., the intracellular lactic acid concentration was higher than in the external medium. Under the conditions used the glucose added would be consumed within the first 30 min. of the experiment. After 3·5 hr. the intracellular concentration of lactic acid was less, and fell faster during the next 1·75 hr., than the concentration outside the cell.

DISCUSSION

Previous investigators of the Crabtree effect have suggested various mechanisms for its explanation. Brin & McKee (1956) have proposed that it is related to the concentration of inorganic phosphate. Racker (1956), Chance & Hess (1956) and Shacter (1955), suggest that the effect is not due solely to a pH change. The evidence against a simple pH change is that methylene blue and dinitrophenol abolish the effect without altering the overall pH change, and iodoacetic acid prevents the pH change without altering the Crabtree effect. Racker (1956) and Wu & Racker (1957) suggested an alternative mechanism involving the cytoplasmic shuttling of an adenine nucleotide. Bloch-Frankenthal & Weinhouse (1957) have pointed out that the point of inhibition must be such as to act on the oxidation of endogenous substrate as well as on the oxidation of glucose, without affecting glycolysis.

Although we have no proof that intracellular pH is responsible for the Crabtree effect, we have shown that the intracellular lactic acid concentration is increased after addition of glucose and, under special conditions, can be higher than in the external medium. We therefore cannot exclude the possibility that an intracellular increase in hydrogen-ion concentration may have some part in the reduction of respiration rate. The fact that the intracellular lactic acid concentration can be very different from the concentration in the external fluid shows that the combined ability of the cell to remove lactic acid by oxidation and to transport it to the external fluid is not large in comparison with the rate of conversion of glucose into lactic acid. Further studies on the removal of lactic acid from the cell are in progress.

SUMMARY

1. Below pH 6·0 the Crabtree effect in Ehrlich ascites tumour cells (i.e. inhibition of respiration caused by addition of glucose) is very small or non-existent. The rate of removal of glucose from solution by the cells is slower at pH 6·0 than at pH 7·4, but is still considerable.

2. As taken from the mouse, Ehrlich ascites tumour cells have little or no lactic acid in them. The lactic acid is essentially all in the ascitic fluid.

3. When excess of glucose is added *in vitro*, the lactic acid content of the cells becomes greater than that of the fluid. Some time after all the added glucose has been used the situation is reversed again and there is a progressive decrease in intracellular lactic acid concentration.

4. The increased intracellular concentration of lactic acid after addition of glucose shows that a relationship between the concurrent increased inhibition of respiration observed and a lowered intracellular pH cannot be excluded.

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The Distribution of Bound Acetylcholine and Choline Acetylase in Rat and Pigeon Brain

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Mann, Tennenbaum & Quastel (1938) presented evidence that about three-quarters of the acetylcholine in guinea-pig brain was bound to some protein constituent of the cell, and later the same authors (1939) suggested that this was a complex of acetylcholine and its synthesizing enzyme. It has since been pointed out (Braganca & Quastel, 1952) that bound acetylcholine could be acetylcholine held within the mitochondria, and from indirect evidence del Castillo & Katz (1956) conclude that intracellular granules containing acetylcholine may function in synaptic transmission.

Hebb & Whittaker (1958) have located bound acetylcholine and choline acetylase in particulate fractions from guinea-pig and rabbit brain. Bound acetylcholine and choline acetylase have also been found in mitochondrial preparations from locust-head tissue (Bellamy, 1958).

This paper records the distribution of bound acetylcholine and choline acetylase in rat- and pigeon-brain homogenates fractionated by differential centrifuging and provides evidence that both the enzyme and its stored product occur in the same particulate fraction.

METHODS

Preparation and fractionation of the homogenates. Brains from rat or pigeon were forced through a chilled stainless-steel Fisher mincer (Jouan, Paris) and homogenized with 4 vol. of 0·25 M-sucrose soln. at 0° in a stainless-steel homogenizer of the Potter–Elvehjem type (working volume 40 ml.). The clearance of the pestle in the cylinder was 0·005 in. and it was moved up and down eight times, revolving at a speed of 2000 rev./min.

The homogenate was diluted with a further 2 vol. of 0·25 M-sucrose soln. and fractionated at the gravitational forces and times described later. Each fraction was examined by phase-contrast microscopy and it was found that the nuclear and microsomal fractions were not entirely free from mitochondria. After sedimentation of each fraction it was washed three times with 0·25 M-sucrose soln. (one-third of the original volume of the tissue mince) and the washings were added to the supernatant fluid from that fraction. The last centrifuging of the fractions was carried out at 25 000 *g* for 15 min. and the tightly packed residue resuspended in 9 vol. of 0·25 M-sucrose soln. The microsomal fraction was not washed but resuspended immediately in 9 vol. of 0·25 M-sucrose soln. All the operations were carried out as close to 0° as possible.

Preparation of electron micrographs. Small samples of the mitochondrial and microsomal pellets were fixed in 1% osmic acid adjusted to 0·25 osmolar with sucrose, then dehydrated and embedded in butylmethacrylate as described by Bellamy (1958). Sections were cut and examined under the electron microscope by Dr G. Meek of the Department of Anatomy, Oxford.

Extraction and estimation of acetylcholine. Acetylcholine in the tissue preparations was extracted with 1% trichloroacetic acid soln. after brief heat treatment as described previously (Bellamy, 1958), except that the ion-exchange resin was not used. The extracted acetylcholine was measured with the frog rectus abdominis-muscle preparation described by Chang & Gaddum (1933), with the controls suggested by Feldberg (1942–43).

Determination of dry weights. The dry weights of tissue samples were determined as acid-insoluble dry matter according to the method of Werkheiser & Bartley (1957).

RESULTS

Distribution of bound acetylcholine in brain tissue. No inhibitor of acetylcholine esterase was added during the preparation of the homogenate or during the fractionation procedure. This ensured that only acetylcholine bound to tissue components was present (Mann *et al.* 1939). A sample of pigeon-brain homogenate contained 13·6 μg. of bound

acetylcholine/g. dry wt. and of rat brain 5·8 μg./g. dry wt.

In rat brain 70 % of the bound acetylcholine was recovered in the mitochondrial fraction against 54 % in pigeon brain (Table 1). The concentration of bound acetylcholine in these fractions was 3½ and 2½ times that of the whole homogenate respectively (18 and 33 μg./g. dry wt.).

About 14 % of the total acetylcholine of the homogenate was recovered in the microsomal fraction of rat brain and the concentration was a quarter of that in the homogenate. The pigeon-brain microsomal fraction contained 7 % of the acetylcholine of the homogenate at a concentration as high as in the mitochondrial fraction.

Nearly 40 % of the acetylcholine of pigeon brain was found in the combined cell debris and nuclear fraction. The concentration was lower than that in the homogenate and microscopic observation showed that this fraction contained more incompletely broken cells than the corresponding fraction from rat brain.

Distribution of enzymes forming acetylcholine. Acetylcholine synthesis is expressed as the total acetylcholine formed/g. dry wt. on incubating the tissue suspension 1 hr. at 38°. The rate of synthesis was not linear over this period; the amount of acetylcholine synthesized in 30 min. was generally two-thirds of that formed after 1 hr.

Eserine sulphate (mM) was added to all incubation mixtures. On incubating the brain homogenates in the presence of adenosine triphosphate (ATP) and choline (Table 2) there was a slight rise in the total acetylcholine. This was augmented on adding acetate, reaching a value of 8·1 μg. and 11·1 μg./g./hr. for rat and pigeon respectively. In the subcellular particulate fractions acetylcholine synthesis occurred only when acetate was included in the incubation medium. The supernatant fraction did not synthesize acetylcholine under the conditions used, a finding which contrasts with that of Hebb & Smallman (1956) on rabbit brain, where 25 % of the enzyme activity appeared in the supernatant fraction.

Table 1. *Distribution of bound acetylcholine in fractionated homogenates of rat and pigeon brain*

Rat brains (8 g. wet wt.) were homogenized in 32 ml. of 0·25 M-sucrose soln. and diluted and fractionated as described in the text. The cell debris was sedimented at 1000 *g* for 10 min., the nuclear fraction at 2000 *g* for 10 min., the mitochondrial fraction at 15 000 *g* for 10 min. and the microsomal fraction at 25 000 *g* for 40 min. Pigeon brains (20 g. wet wt.) were homogenized and fractionated as described above except that the mitochondrial fraction was sedimented between 4000 and 14 000 *g* in 15 min.

	Acetylcholine content of tissues			
	Rat (μg. in fraction)	Pigeon (μg. in fraction)	Rat (μg./g. dry wt.)	Pigeon (μg./g. dry wt.)
Whole homogenate	14·1	46·0	5·4	13·6
Fractions:				
Cell debris and nuclei	2·9	18·3	5·8	9·9
Mitochondria	9·8	25·0	18·7	33·2
Microsomes	2·2	3·3	1·2	32·8
Supernatant	0	0	—	—
Recovery	14·9	46·6	—	—

Table 2. *Synthesis of acetylcholine by fractionated brain homogenates*

Homogenates were fractionated as described in Table 1. Incubation medium: 4 ml., containing 0·017 M-potassium phosphate buffer, pH 7·4, 0·013 M-$MgCl_2$, 2 mM-ATP, 0·033 M-choline chloride and mM-eserine sulphate. Acetate was added to a final concn. of 0·033 M where indicated. After addition of the particle suspension (2 ml. containing 100 mg. dry wt.) the medium was adjusted to 0·25 osmolar with sucrose, and the incubation was carried out in air at 38° for 1 hr.

	Total acetylcholine synthesized (μg./g. dry wt./hr.)			
	Rat		Pigeon	
	Endogenous	With acetate	Endogenous	With acetate
Homogenate	3·3	8·1	1·5	11·7
Fraction:				
Cell debris and nuclei	0	1·5	0	1·3
Mitochondria	0	27·1	0	76·5
Microsomes	0	12·4	0	60·7
Supernatant	0	0	0	0

The rate of acetylcholine formation in the mitochondrial fraction was greater than in the homogenates. It was three times greater in the rat and seven times greater in the pigeon (27 and 76 μg./g. dry wt./hr. respectively). The increase in the total amount of acetylcholine in both rat- and pigeon-brain mitochondrial fractions after incubating them for 1 hr. at 38° (in the presence of acetate, ATP and choline) was threefold (Table 3). As already mentioned no synthesis occurred if acetate was omitted and a considerable amount of the bound acetylcholine was released into the surrounding medium (64 % for pigeon and 46 % for rat). The bound acetylcholine of the particles fell on incubation with choline and ATP. In rat-brain particles the amount of bound acetylcholine fell during incubation whether acetate was present or not. In pigeon brain the addition of acetate maintained the initial acetylcholine content of the particles.

The microsomal fraction also formed acetylcholine faster than the homogenate. This was most marked in the pigeon, where the activity of the enzymes in the microsomal fraction was nearly as high as in the mitochondrial fraction. In pigeon brain all of the activity of the homogenate was recovered in the derived fractions (Table 4). About 53 % of the total activity was found in the mitochondrial fraction. On fractionation of rat-brain homogenate only 60 % of the total enzyme activity was recovered in the derived fractions.

Relationship between bound acetylcholine and enzymes forming acetylcholine. The distribution of bound acetylcholine between the fractions derived from pigeon brain was the same as that of the enzymes forming acetylcholine (Table 4). The results indicate that the mitochondrial and microsomal fractions are the main binding sites.

Electron micrographs of sections through these fractions show particles with a typical mitochondrial structure (Witter, Watson & Cottone, 1955; Lever & Chappell, 1958). Small unidentified vesicles distinct from mitochondria were seen in both mitochondrial and microsomal fractions, and from this evidence it is impossible to say that one type of subcellular particle only is associated with bound acetylcholine and choline acetylase.

Since the completion of this work, Whittaker (1958) has reported the separation of particles rich in acetylcholine and choline acetylase from particles containing the oxidative enzymes thought to be characteristic of mitochondria. The separated particles are structurally distinct from mitochondria and appear to be similar to the unidentified vesicles seen in the particulate fractions from rat and pigeon brain.

DISCUSSION

The localization of choline acetylase within the nerve cells bears on the question of the physiological role of acetylcholine in the transmission of impulses. However, as it is not yet possible to correlate the isolated particles containing acetylcholine and choline acetylase with known structures of the nerve cells no inferences can be made from the present findings about the function of acetylcholine.

Table 3. *Synthesis of acetylcholine in the mitochondrial fractions prepared from rat and pigeon brain*

The mitochondrial fractions were incubated as in Table 2. After 1 hr. the mitochondria were isolated from the incubation mixture by centrifuging at 14 000 *g* for 15 min. The sedimented particles were washed once with 10 vol. of 0·25 M-sucrose soln. and the acetylcholine in the particles and the supernatant medium was determined.

	Total acetylcholine in the incubation mixture (μg./g. dry wt.)		Percentage distribution of acetylcholine in the incubation mixture			
			Mitochondria		Suspending medium	
Additions	Rat	Pigeon	Rat	Pigeon	Rat	Pigeon
Nil	15·1	37·7	54	36	46	64
0·033 M-Acetate	42·1	113	19	39	81	61

Table 4. *Recovery of bound acetylcholine and acetylcholine-forming enzymes of the whole homogenate*

Figures are given as the percentage of the total amount of bound acetylcholine and enzymes forming acetylcholine (choline acetylase) of the homogenate recovered in each fraction.

	Rat		Pigeon	
	Acetylcholine	Choline acetylase	Acetylcholine	Choline acetylase
Cell debris and nuclei	21	20	40	44
Mitochondria	70	34	54	53
Microsomes	16	6	7	7
Supernatant	0	0	0	0
Total recovery	107	60	101	104

One of the problems is the mechanism which controls the quantities of acetylcholine synthesized under physiological conditions. The synthesis is probably not continuous but, during the resting state of the tissue, comes to a standstill when certain amounts have accumulated. The key substances required for synthesis in the intact tissue include acetyl-coenzyme A and choline. Acetyl-coenzyme A cannot be a controlling factor since it is produced in relatively large quantities by the normal oxidative metabolism. Nor is the amount of choline likely to be a limiting factor because the level of free choline in rat brain has been estimated at 1·4 μmoles/g. fresh weight (Leucke & Pearson, 1944), which is about 200 times the average level of total acetylcholine in the brain. The present experiments show some parallellism between the rate of acetylcholine synthesis, the capacity of brain particles for binding acetylcholine and the rate of release of the bound ester, in that all are greater in the pigeon brain than in the rat brain. This might be taken to suggest that the enzyme system also provides the binding site and that the enzyme system becomes inactivated when the binding sites are saturated with acetylcholine.

SUMMARY

1. Homogenates of rat and pigeon brain in 0·25 M-sucrose solution have been fractionated by differential centrifuging and the distribution of bound acetylcholine and choline acetylase in the fractions has been determined.

2. The mitochondrial fractions from rat and pigeon brain contained respectively 70 % and 54 % of the total bound acetylcholine of the whole homogenate (on a dry-weight basis these figures correspond to 18 and 33 μg./g.).

3. Choline acetylase activity was detected in all fractions of the homogenate except the supernatant fractions. Activities of the enzymes (per g. dry wt.) in the mitochondrial and microsomal fractions were higher than the activity of the respective homogenates. For rat- and pigeon-brain mitochondrial fractions the respective rates were 27 μg. and 76 μg./g. dry wt./hr. at 38°, equivalent to three and seven times those in the whole homogenates.

4. On incubating the brain-mitochondrial suspension with choline no synthesis of acetylcholine occurred but between 64 % (pigeon brain) and 46 % (rat brain) of the bound acetylcholine was released into the surrounding medium. On incubation of pigeon-brain particles with choline plus acetate a synthesis of acetylcholine took place and the initial level of bound acetylcholine was maintained.

5. The rate of acetylcholine synthesis, the capacity of the particles for binding acetylcholine and the rate of release of the bound ester were all greater in pigeon brain than in rat brain.

The author wishes to thank Professor Sir Hans Krebs, F.R.S., for his helpful criticism during the preparation of the manuscript and Dr W. Bartley for much discussion and advice during the experimental work. The work was carried out during the tenure of a Medical Research Council Scholarship for training in research methods, and was aided by a grant from the Rockefeller Foundation.

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Studies on Alkaline Phosphatases

1. KINETICS OF PLASMA PHOSPHATASE OF NORMAL AND RACHITIC CHICKS

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In a previous paper some of the general characteristics of the plasma phosphatase of chicks have been described (Motzok & Wynne, 1950). It was established also that the phosphatase activities of plasma and of bone extracts increased markedly with decreasing dosages of vitamin D, but activities of extracts of liver, kidney and intestinal mucosa were not related to the vitamin D intake (Motzok, 1950). The increase in plasma phosphatase in rachitic conditions has been regarded as the result of leakage of the enzyme from the bones into the blood stream. It was of interest to determine whether or not vitamin D administered to the chick had an effect on the alkaline phosphatase of the plasma in a manner that would alter the affinity of the enzyme for a substrate when the activity was measured *in vitro*.

This paper is concerned with the applicability of the Michaelis & Menten (1913) theory of enzyme kinetics to the activities of the plasma phosphatases of normal and rachitic chicks and with the influence of the relationship between substrate concentration and the optimum pH for the enzyme activity on the K_m (Michaelis constant) of the enzyme–substrate complex. This study, using glycerol β-phosphate (β-glycerophosphate) as the substrate, was completed in 1944 and was the subject of a thesis (Motzok, 1945). Subsequently, the hydrolysis of phenyl phosphate by plasma phosphatase of mature fowls was investigated. Studies were made on the variation of optimum pH with substrate concentration, the influence of pH on K_m and the determination of K_m with the optimum pH for each concentration of substrate. Variations in pK_m with pH, when each value for K_m was determined at a constant pH, are also discussed.

METHODS AND MATERIALS

The rearing of normal and rachitic chicks and the methods for the preparation of plasma and the determination of phosphatase activity have been described previously (Motzok & Wynne, 1950). Plasma from mature fowls, fed on a commercial diet, was used in the studies with disodium phenyl phosphate [$Na_2(C_6H_5)PO_4$]. In the latter experiments the liberated phenol was determined as the measure of phosphatase activity by a modification (Kaplan & Narahara, 1953) of the Gomori method (1949).

EXPERIMENTAL AND RESULTS

Experiments with β-glycerophosphate

Influence of pH *and substrate concentration on phosphatase activity.* Data in Fig. 1 show that the initial concentration of substrate had a marked influence on the pH required for optimum activity of chick plasma phosphatase; the optimum shifted from pH 9·5 for mM-sodium β-glycerophosphate to pH 10·0 for 0·12 M-substrate in mixtures containing 5 mM-$MgCl_2$. The determination of pH optima with higher concentrations of this substrate is subject to an error caused by precipitation in the reaction mixtures at the high pH values, since these are close to the effective limits of the buffer system.

This change in optimum pH for alkaline phosphatases with change in initial concentration of substrate is in general agreement with the limited findings of other workers (see Motzok & Wynne, 1950), which have been confirmed for a wide range of substrate concentration by Ross, Ely & Archer (1951) and Morton (1957). A similar relationship

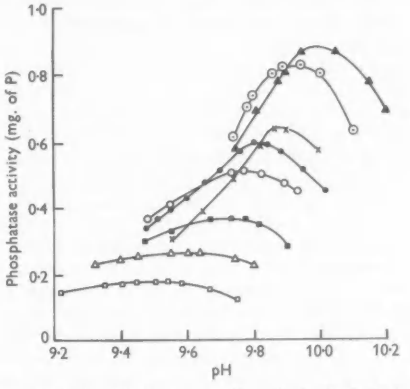

Fig. 1. Relationship between pH and the hydrolysis of different concentrations of sodium β-glycerophosphate (□, 1 mM; △, 2 mM; ■, 6 mM; ○, 0·01 M; ●, 0·02 M; ×, 0·03 M; ⊙, 0·08 M; ▲, 0·12 M) by chick plasma phosphatase in the presence of 5 mM-$MgCl_2$, during a reaction period of 10 min.

between optimum pH and substrate concentration was exhibited in the activity of the acid phosphatase of yeast (Dr A. M. Wynne, personal communication).

Fig. 1 also shows that with low concentrations of substrate maximum activity was obtained over a wider range of pH than with high concentrations of substrate. Moreover, when the activities were measured at a pH on the acid side of the optimum for a high concentration of substrate, the rate of hydrolysis was not as great as when the initial concentration was lower. This illustrates the phenomenon which many workers have attributed to 'inhibition' by excess of substrate and shows the effect of pH in the manifestation of this characteristic of phosphatases. For example, at pH 9·55 (the optimum pH for 2 mM-substrate), the rates of hydrolysis increased with increasing concentration of substrate up to 0·01 M. The rate with 0·02 M-substrate at pH 9·55 was slightly lower than that with 0·01 M; with 0·03 M-substrate the rate of hydrolysis at this pH was lower than the rate with 6 mM and considerably below that with 0·01 M-substrate. On the other hand, a comparison of activities at the optimum pH for each concentration of substrate shows that there was no diminution in rates of reaction with high concentrations of substrate up to 0·12 M.

The importance of the use of short periods of reaction in the determination of optimum pH has been emphasized (Motzok & Wynne, 1950), particularly for phosphatases, since variations in the initial concentration of substrate affects the optimum pH.

Influence of pH *on* K_m. Folley & Kay (1935) concluded from their studies that the relationship between the concentration of monophenyl phosphate and its hydrolysis by mammary-gland phosphatase conformed to the theory of Michaelis & Menten (1913). Similar observations have been made by Jacobsen (1933), Kutscher & Wörner (1936), Pfankuch (1936), Bailey & Webb (1944), Bodansky (1946) and Schønheyder (1951) in their investigations with other phosphatases.

Previous workers, in determining the K_m of phosphatases, have adjusted all reaction mixtures (containing increasing concentrations of substrate) to the same pH, namely, an 'optimum' pH value for an arbitrarily chosen concentration of substrate. Often a pH which is optimum for a relatively high concentration of substrate has been used. Fig. 1 shows that at a high pH the hydrolysis of the substrate in low concentration is inhibited by hydroxyl ions and the determination of K_m under these conditions would be influenced by this inhibition.

The nature of the inhibition, competitive or non-competitive, was studied by plotting data according to the method of Lineweaver & Burk (1934). Three concentrations of enzyme were used, namely, 0·375, 0·5 and 0·75 ml. of plasma/20 ml. of digest for each set of concentrations of substrate (1·2 mM to 0·08 M). The pH was maintained at 9·88 and the concentration of added Mg^{2+} ions was 5 mM in the digest; this amount of Mg^{2+} ions was found to be optimum for high concentrations as well as dilute solutions of substrate. Under these conditions the concentration of inhibitor (hydroxyl ions) remained constant, but the amount of enzyme, hence the inhibitor:enzyme ratio, was varied.

The line of best fit for each set of values in Fig. 2 was determined by the method of least squares (Treloar, 1939) and the values for K_m and $V_{max.}$ were derived from these calculations. These show that K_m values obtained from velocities at a constant pH, the optimum for a high concentration of substrate, decreased with increasing amounts of enzyme; with 0·375 and 0·75 ml. of plasma/20 ml. of digest the values for K_m were 37·9 and 19·5 mM respectively. Fig. 2 also shows that the inhibition by hydroxyl ions did not appear to be perfectly competitive in nature. $V_{max.}$ increased from 1·14 to 1·59 when the concentration of enzyme was increased twofold. There is no doubt that some of the discrepancies in the value for K_m for the phosphatase of the same source, which have been reported in the literature, are due in part to variations in the amount of enzyme employed and the inhibitory effect of hydroxyl ions when the hydrolysis of glycerophosphate, present in low concentration, was measured at a relatively high pH.

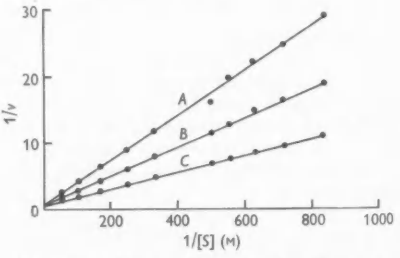

Fig. 2. Effect of dilution of plasma phosphatase on K_m when the velocities were measured at pH 9·88 for all concentrations of sodium glycerophosphate (mixture of α and β forms). Curves *A*, *B* and *C* were obtained with 0·375, 0·5 and 0·75 ml. of plasma/20 ml. of digest in the presence of 5 mM-$MgCl_2$, during a reaction period of 10 min. Data were plotted according to Lineweaver & Burk (1934).

Enzyme concentration	$V_{max.}$	K_m (M)
A	1·14	0·0379
B	1·24	0·0270
C	1·59	0·0195

The phosphatase activity of plasma of chicks fed on a vitamin D-free diet was found to be about twice that of plasma from chicks receiving 20 units of vitamin D/100 g. of diet. Fig. 3 shows that the slope of the $1/v:1/[S]$ line for the enzyme in plasma of chicks fed with 20 units of vitamin D/100 g. of ration is considerably greater and K_m smaller than those for the enzyme of plasma of chicks reared on the vitamin D-free diet. These relationships are analogous to the effect of dilution of the enzyme (Fig. 2).

The establishment of the relationship between optimum pH and substrate concentration for alkaline phosphatase, illustrated in the previous section, suggested the maintenance of optimum pH for each dilution of substrate in the determination of K_m of alkaline phosphatase of chick plasma.

Five preparations of plasma were used, one from 40 chicks fed on a vitamin D-free diet, three dilutions of plasma obtained from 120 chicks which received 10 units of vitamin D/100 g. of diet and one preparation of plasma from 40 chicks which received 20 units of vitamin D/100 g. of diet. The Mg^{2+} ion concentration was maintained at 5 mM in all reaction mixtures.

Plots of experimental values (Fig. 4) showed that with 1·2–4·0 mM-substrate the $1/v:1/[S]$ relationships were linear. The values for K_m and $V_{max.}$, designated as K_{m_1} and $V_{max.1}$, were derived from the calculations of the lines of best fit for this range of substrate concentration. With concentrations of substrate from 6 mM to 0·02 M, the $1/v:1/[S]$ relationships varied in a curvilinear manner. With substrate concentrations greater than 0·02 M the $1/v:1/[S]$ relationships were again linear, but the slopes of the lines of best fit were considerably greater than for those obtained with the low concentrations of substrate. These data were used to calculate the second set of values for K_m and $V_{max.}$, designated as K_{m_2} and $V_{max.2}$, for the hydrolysis of substrate present in high concentration (Fig. 5).

These data show that the inhibition by hydroxyl ions, causing variation in K_m with changes in enzyme concentration when all reactions were measured at a relatively high pH, e.g. pH 9·9, appeared to be eliminated when the optimum pH for each concentration of substrate was used.

At this stage of the investigation it was found that the glycerophosphate, which had been used as substrate, contained about 33 % of the α form and 67 % of the β form of the compound. A sample of pure β-glycerophosphate became available and experiments were conducted to confirm the findings

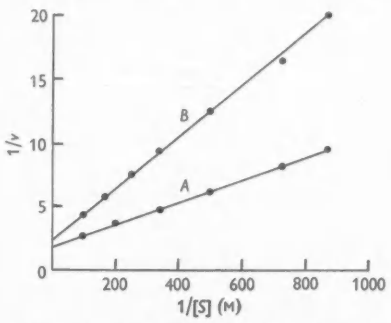

Fig. 3. K_m values for the hydrolysis of sodium glycerophosphate (mixture of α and β forms) by the phosphatase of plasma (0·5 ml./20 ml. of digest) obtained from chicks fed on a vitamin D-free diet (curve *A*) and from chicks fed with 20 units of vitamin D/100 g. of diet (curve *B*). Hydrolysis was performed at pH 9·9 for all concentrations of substrate in the presence of 5 mM-$MgCl_2$, during a reaction period of 10 min. Data were plotted as in Fig. 2.

Enzyme preparation	$V_{max.}$	K_m (mM)
A	0·72	6·2
B	0·42	9·6

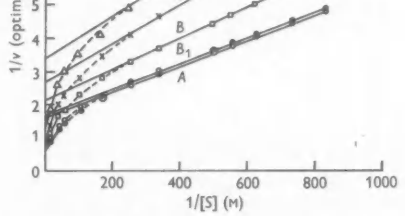

Fig. 4. K_m values (designated as K_{m_1}) for the hydrolysis of dilute solutions of sodium glycerophosphate (mixture of α and β forms) by chick plasma phosphatase at the optimum pH for each concentration of substrate in the presence of 5 mM-$MgCl_2$, during a reaction period of 10 min. Curves *A*, *B* and *C* were obtained with three samples of plasma (0·5 ml./20 ml. of digest) from three groups of chicks which had been fed with 0, 10 and 20 units of vitamin D/100 g. of diet respectively. Curves B_1 and B_2 were obtained with 0·75 and 0·375 ml. of plasma (used for curve *B*)/20 ml. of digest. Data were plotted as in Fig. 2.

Enzyme preparation	$V_{max.1}$	K_{m_1} (mM)
A	0·64	2·4
B	0·47	2·2
B_1	0·61	2·2
B_2	0·30	1·8
C	0·38	2·1

described above. The shift in optimum pH with changes in the initial concentration of pure β-glycerophosphate was the same as with the mixture of α and β salt (Motzok, 1945). When the optimum pH was employed for each concentration of substrate, the $1/v:1/[S]$ relationships were similar to those shown in Figs. 4 and 5. The calculated K_m values for two dilutions of the enzyme (0·375 and 0·5 ml. of plasma/20 ml. of digest) were 2·1 and 2·2 mM with the dilute solutions of pure β-glycerophosphate and 0·03 M with the high concentrations of this substrate.

Experiments with phenyl phosphate

Influence of pH *and substrate concentration on phosphatase activity.* The rate of hydrolysis of disodium phenyl phosphate by plasma phosphatase of fowls was determined in the presence of 5 mM-$MgCl_2$ at sufficient values of pH to establish the optimum pH for a wide range of substrate concentration. The data in Fig. 6 (*A*) show that the optimum shifted from pH 9·4 for the hydrolysis of mM-substrate to pH 10·36 for 0·03 M-substrate.

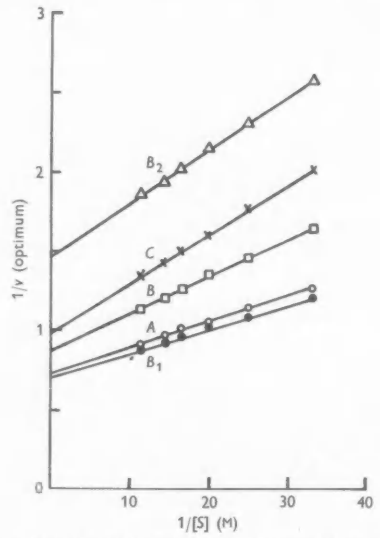

Fig. 5. Part of the data from Fig. 4 expanded to show clearly the $1/v:1/[S]$ relationship and K_m values, designated as K_{m_2}, for the hydrolysis of substrate present in high concentration.

Enzyme preparation	$V_{max\cdot 2}$	K_{m_2} (M)
A	1·38	0·021
B	1·15	0·026
B_1	1·45	0·023
B_2	0·69	0·023
C	1·01	0·030

The pH–activity curves shown in Fig. 6 (*B*) were derived from the data presented in Fig. 7. This selection of the data was made to show clearly the rates of reactions with concentrations of substrate from 2 mM to 0·03 M on the acid side of the pH optima and to illustrate the phenomenon described by other workers as inhibition by excess of substrate. At pH 8·9 the highest rate was obtained with 2 mM-substrate and the lowest rate with 0·03 M-substrate. With changes in pH toward more alkaline conditions the differences in the rates diminished. At pH 9·7 the rates were approximately equal with 0·01, 0·02 and 0·03 M-substrate and appreciably higher than the rates with 2 and 5 mM-substrate. At still higher pH values the greatest velocity was obtained with the highest concentration of substrate.

These data also show that with fowl plasma phosphatase the pH optima for the various concentrations of phenyl phosphate were appreciably

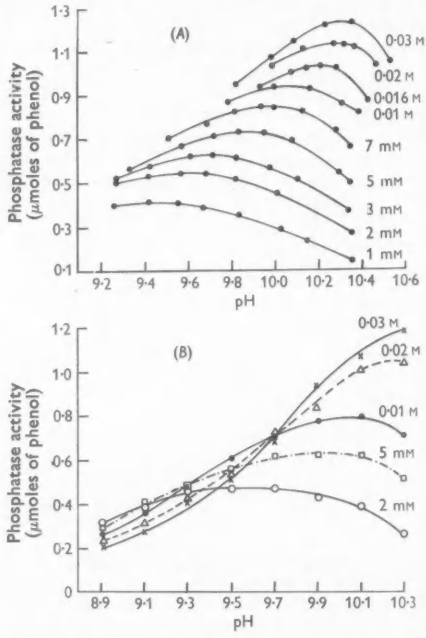

Fig. 6. (*A*) Relationship between pH and the hydrolysis of different concentrations of disodium phenyl phosphate by fowl plasma phosphatase. (*B*) Data derived from Fig. 7, showing the relationship between rates of hydrolysis and initial concentrations of substrate on the acid side of pH optima. Activities are expressed as phenol (μmoles) liberated by the enzyme in 1 ml. of plasma in the presence of 5 mM-$MgCl_2$, during a reaction period of 15 min.

higher than the pH optima for the corresponding concentrations of glycerophosphate (Fig. 1).

Influence of pH *on* K_m. The effect of pH on K_m of fowl plasma phosphatase was determined with disodium phenyl phosphate, 16 dilutions of the substrate being used, from mM to 0·03 M, at eight pH values from 8·9 to 10·3, and plotting the data (Fig. 7) according to the method of Lineweaver & Burk (1934). At pH 8·9 and 9·1 the reciprocals of velocities with high concentrations of substrate deviated markedly from linearity and only the data with 1–3 mM-substrate were used to calculate the lines of best fit; at other pH values the data for 1–4 mM-substrate were employed. The values for K_m (Fig. 7) increased with increasing alkalinity from 1·5 mM at pH 8·9 to 8·3 mM at pH 10·3.

The rates of hydrolysis of phenyl phosphate were also determined at the optimum pH for each concentration of substrate. Fig. 8 shows that with 1–3 mM-substrate the $1/v:1/[S]$ relationship was linear and these values were used in the calculation of the line of best fit and K_{m_1} for dilute substrate. The slope of the line (A) was 0·00125 and K_{m_1} was mM. The transition point in the $1/v:1/[S]$ relationship from the low to the high concentrations of substrate was at approximately 4 mM-substrate. With concentrations of substrate greater than 4 mM the $1/v:1/[S]$ relationship was also linear but the slope of the line (B) was 0·0033 and K_{m_2} derived from these data was 4·5 mM.

Influence of pH *on* pK_m. When the values for K_m for phenyl phosphate, determined previously (Fig. 7), were plotted as pK_m versus pH, they

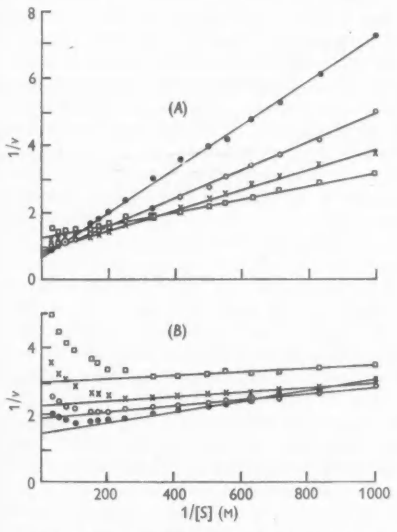

Fig. 7. The influence of pH on K_m values for the hydrolysis of disodium phenyl phosphate by fowl plasma phosphatase. Activities were expressed as in Fig. 6 and plotted as in Fig. 2. K_m values (mM): (A) ●, 8·3 (pH 10·3); ○, 5·8 (pH 10·1); ×, 3·0 (pH 9·9); □, 1·6 (pH 9·7). (B) ●, 0·95 (pH 9·5); ○, 0·50 (pH 9·3); ×, 0·27 (pH 9·1); □, 0·15 (pH 8·9).

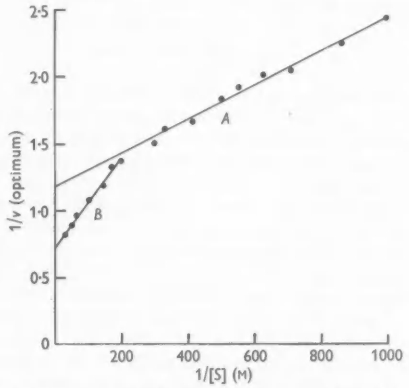

Fig. 8. K_m values (designated as K_{m_1} for dilute substrate and K_{m_2} for substrate present in high concentration) for the hydrolysis of disodium phenyl phosphate by fowl plasma phosphatase when the activities were determined at the optimum pH for each concentration of substrate. Activities were expressed as in Fig. 6 and plotted as in Fig. 2. Curve A, K_{m_1} 1·1 mM; curve B, K_{m_2} 4·5 mM.

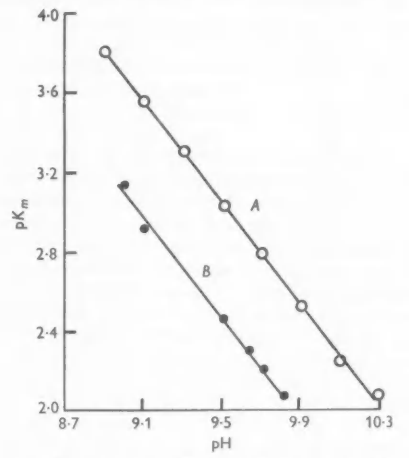

Fig. 9. Effect of pH on pK_m of alkaline phosphatases. Curve A is for fowl plasma phosphatase with phenyl phosphate (data from Fig. 7). Curve B is for bone phosphatase of rachitic chicks with β-glycerophosphate.

appeared to fall along a straight line with a slope of $-1{\cdot}3$ (Fig. 9, curve *A*). A similar relationship between pK_m and pH (Fig. 9, curve *B*) was obtained with data (unpublished) from an earlier study on bone phosphatase of rachitic chicks with β-glycerophosphate as the substrate. In both cases the velocities were measured in the presence of the optimum concentration of Mg^{2+} ions (5 mM) in the reaction mixtures.

DISCUSSION

In the determination of rates of reaction for the calculation of K_m, the general practice has been to maintain a constant pH for all concentrations of substrate. This procedure is applicable to enzyme systems which exhibit pH–activity curves of similar shape, and have optimum activity at the same pH for all concentrations of substrate. Any effect of pH can be avoided by the simple expedient of measuring velocities at the optimum pH.

With phosphatases, however, the data in Figs. 1 and 8 and the findings of Motzok & Wynne (1950), Ross *et al.* (1951) and Morton (1957) show that the pH–activity curves are not similar in shape for all concentrations of substrate and that the optimum pH varies with changes in the initial concentration of substrate. Indeed, there appears to be no single value of pH at which hydrogen or hydroxyl ions do not have some inhibitory effect on phosphatase activity when the range of concentration of substrate is large enough for an accurate estimation of K_m.

Many workers (see Laidler, 1955) have considered the influence of pH on the rate of enzyme reactions and various types of equations have been devised for the analysis of specific conditions. With phosphatases, however, the influence of pH is quite complex and no one has proposed a solution of the problem.

With enzyme systems in which the optimum pH does not vary with changes in the initial concentration of substrate, the observed velocities are in fact 'optimum' velocities for each concentration of substrate when the activities are measured at the optimum pH. The only way in which comparable 'optimum' velocities can be obtained for phosphatases is by measuring activities at the optimum pH for each concentration of substrate. It is recognized that in the double-reciprocal plot of the data, shown in Figs. 4 and 8, pH is a variable factor. Nevertheless, the velocities were optimum for each concentration of substrate and data derived in this manner gave reasonably constant values for K_m regardless of the concentration of enzyme in the digestion mixtures.

No doubt some of the discrepancies in the value for K_m for the phosphatase of the same source, reported in the literature, are due in part to variations in the amount of enzyme employed and the inhibiting effect of hydroxyl ions when the hydrolysis of dilute substrate was measured at a relatively high pH. If the inhibition by hydroxyl ions had not been taken into consideration in these studies, the apparently erroneous conclusion might have been drawn that vitamin D, administered to chicks in the basal diet, affected the nature and properties of plasma phosphatase in a manner which altered the K_m of the enzyme–substrate complex (Fig. 3).

The double-reciprocal plot of velocities at the optimum pH for each substrate concentration resolved the data into two parts from which two values for K_m and $V_{max.}$ could be calculated, one set of values from data with dilute substrate and the second from velocities when the substrate was present in high concentrations. The change in the slope of the $1/v:1/[S]$ lines from the low to the high concentrations of glycerophosphate occurred in a curvilinear manner over a range of 6 mM- to 0·02 M-substrate. With phenyl phosphate the change in the slope of the lines took place over a very small range of concentration of substrate, giving no marked curvilinear transition such as the one observed with glycerophosphate.

Neilands & Stumpf (1955) pointed out that, instead of straight lines in the double-reciprocal plot, deviations may be observed with high concentrations of substrate. They suggested that when the substrate concentration is high an enzyme may require two molecules of substrate, and the attachment of one facilitates the rate of reaction with the second molecule, resulting in abnormally high velocities. With phosphatases 'abnormally' high velocities with high concentrations of substrate were evident only when the velocities were measured at the optimum pH for each concentration of substrate (Figs. 4 and 8). If phosphatase has two sites for the substrate, one might expect that $V_{max.}$ calculated from the $1/v:1/[S]$ plot of velocities with high concentrations of substrate (designated here as $V_{max.2}$) would be twice as large as $V_{max.}$ calculated from velocities with dilute substrate (designated as $V_{max.1}$). Values for $V_{max.1}$ and $V_{max.2}$ were calculated (unpublished data) in all cases when K_m was determined, with the optimum pH for each concentration of substrate, and the average of $V_{max.2}/V_{max.1}$ was found to be 1·84. Although this average value approximates the theoretical ratio of 2, there was considerable variation among these ratios, the range being 1·2–2·9. Unless there is an explanation for the wide variation in the degree to which one molecule aids in the rate of reaction with the second molecule, the present data do not support the suggestion that phosphatases can utilize two molecules of substrate at one time when

the substrate concentrations are high and the velocities are measured at the respective optimum pH.

Neilands & Stumpf (1955) also suggested that abnormally slow velocities (similar to those shown in Fig. 7*B*) may cause choking of the active site by two molecules of substrate, a phenomenon described by other workers as inhibition by excess of substrate. Figs. 1 and 6 (*B*) show that the inhibition by 'excess' of substrate occurred at pH values on the acid side of pH optima required for high concentrations of substrate. These data are similar to those of Morton (1957), who used phenyl phosphate with purified calf intestinal phosphatase, and those of Ross *et al.* (1951), who used β-glycerophosphate with a partially purified enzyme of intestinal mucosa of rats.

There have been several reports on the possible occurrence of two or more phosphatases in an organ or tissue. For example, Tsuboi, Wiener & Hudson (1957) isolated a highly purified acid phosphatase of yeast, which appeared to be homogeneous by ultracentrifuging but contained at least four catalytically active components distinguishable by electrophoretic analysis. Although these active components may have existed as such in the yeast, it is possible that they were derived as a consequence of the purification procedure. Gryder, Friendenwald & Carlson (1955) reported on the presence of two alkaline phosphatases in rat kidney based on inhibition by iodoacetate and activation by glycine and zinc, and Trubowitz, Feldman, Benante & Kirman (1957) suggested the occurrence of two alkaline phosphatases in human erythrocytes on the basis of inhibition by ethylenediamine and activation by zinc and magnesium. Both of the studies, however, did not exclude the possibility that treatment with iodoacetate or ethylenediamine altered the original properties of the enzyme with respect to activation by an amino acid or various metals.

The phenomenon, illustrated in Figs. 4 and 8, has been exhibited by the alkaline phosphatases of various tissues (bone, kidney, liver and intestinal mucosa) of several species of mammals and birds (Motzok & Branion, 1959). If the resolution of data into two parts by the double-reciprocal plot of 'optimum' velocities signifies the presence of two enzymes, it would have to be assumed that two phosphatases exist in a variety of tissues and sources, one being able to act with dilute substrate at low pH values extending to the physiological range (Ross *et al.* 1951), and the second characterized only by its requirements *in vitro* of high concentrations of substrate and high pH. The postulation of the second phosphatase cannot be based on an assumption that its properties *in vitro* are functional under physiological conditions. It would seem unlikely that there exists in various tissues an auxiliary enzyme system which would operate when the substrate was present in such high concentrations at an alkaline pH far removed from the physiological range. It is possible, however, that the natural substrate of the second enzyme and the conditions for normal activity may be quite different from those used in these studies.

Although the present data do not explain the 'abnormally' high velocities with high concentrations of substrate measured at each optimum pH, the relative uniformity of the values for K_{m_1} and K_{m_2} for the phosphatase of any one source suggests that constants derived in this manner may have some significance in the kinetics of these enzymes. Similar studies with other enzymes which exhibit relationships between substrate concentration and optimum pH, e.g. urease (Van Slyke, 1942) and β-glucosidase (Hofstee, 1955), may aid in solving the questions raised in the present work on phosphatases.

Van Slyke (1942) has discussed the characteristics of the two-phase reaction of hydrolytic enzymes and has suggested that, when the substrate is present in low concentration, the rate of enzyme action is determined by the velocity of combination of substrate with the enzyme. When the concentration of substrate is large, the rate of combination of substrate with the enzyme becomes negligible compared with that required for the decomposition of the substrate.

Motzok (1945) found that when the initial concentration of substrate was high the rate of reaction was of zero order during the reaction periods used. When the initial concentrations of substrate were low the rate of reaction was found to be unimolecular. Consequently, when the 'optimum' velocities were employed in the determination of K_m, the values for K_{m_1} were derived from velocities which appeared to conform to the unimolecular rate of reaction and the values for K_{m_2} were obtained with reaction rates which were of zero order or true initial velocities.

It may be that, when the velocities are measured at the optimum pH for each concentration of substrate, the value for K_{m_1}, obtained with dilute substrate, gives an estimate which approaches the true value of the dissociation constant of the enzyme–substrate compound (ES), i.e. k_2/k_1, where $E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES$ according to Michaelis & Menten (1913). On the other hand, K_{m_2}, obtained with high concentrations of substrate, may be governed largely by the velocity of decomposition of the enzyme–substrate complex into products of hydrolysis (P) and free enzyme, i.e. K_{m_2} equals $(k_2 + k_3)/k_1$, where $E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \overset{k_3}{\rightarrow} P + E$ according to Briggs & Haldane (1925).

Variation of pK_m *with* pH. Dixon (1953) plotted K_m values, as pK_m versus pH, from the data of Jacobsen (1933) on kidney phosphatase with glycerophosphate and those of Morton (1952) on purified intestinal phosphatase with phenyl phosphate, and obtained straight lines with a slope of $-1{\cdot}0$. He suggested that the discontinuity in the slope of the line for Morton's data, as well as for the data of Folley & Kay (1935) on milk phosphatase with phenyl phosphate, may be due to an ionizing group in the enzyme concerned with the combination of phenyl phosphate but not with glycerophosphate.

In the present study there was no evidence of discontinuity of the slope of the line of pK_m–pH plots of data with either glycerophosphate or phenyl phosphate. The data in Fig. 9 also did not appear to fit precisely Dixon's theory of a -1 unit relationship between pK_m and pH, the slope of the lines being $-1{\cdot}3$. These K_m values were derived from velocities measured in the presence of the optimum concentration of magnesium chloride (5 mM). Preliminary studies of the role of Mg^{2+} ions on the reaction constant have shown that, when no magnesium chloride was added to the plasma phosphatase–phenyl phosphate system, the slope of the line of the pK_m–pH plot was only about $-0{\cdot}8$ (unpublished data). Indeed, Folley & Kay (1935) used mM-magnesium chloride in the reaction mixtures and Jacobsen (1933) used 2·4 mM-magnesium chloride. Although, Morton (1957) employed a concentration of 0·01 M-magnesium chloride for intestinal phosphatase, the enzyme was highly purified. Furthermore, Morton's data produced a non-linear relationship when plotted as $1/v:1/[S]$ and he estimated K_m values from Michaelis–Menten plots as the substrate concentration for one-half of maximum velocity (1957). Determination of K_m by the latter procedure may be subject to an appreciable error, particularly when the data do not conform to the Lineweaver & Burk derivation of the Michaelis–Menten equation (1913). Morton (1957) observed no substantial change in optimum pH with and without added magnesium chloride and he suggested that the deviation from linearity in the $1/v:1/[S]$ plots was due to competitive pathways in the formation of the enzyme–metal–substrate complex as postulated by Friedenwald & Maengwyn-Davies (1954).

The influence of Mg^{2+} ions on the pK_m–pH relationship for plasma phosphatase may be due at least in part to its effect on the pH–activity characteristics of this enzyme with different concentrations of substrate. It was found that, when no magnesium chloride was added, the pH optima shifted to lower values with β-glycerophosphate (Motzok, 1945) and phenyl phosphate (Motzok & Branion, 1959) from the pH optima in the presence of added magnesium chloride. In addition, without added magnesium chloride, optimum activity was observed over a wider range of pH, particularly with higher concentrations of substrate than with added magnesium chloride. K_m values, determined at constant pH, would be affected by variation in the inhibition by hydroxyl ions with different concentrations of substrate when the amount of added magnesium chloride was varied.

SUMMARY

1. The optimum pH for the hydrolysis of β-glycerophosphate and phenyl phosphate by fowl plasma phosphatase was found to change to higher values with increases in the initial concentration of substrate. The pH optima for various concentrations of phenyl phosphate were higher than the optima for the corresponding concentrations of β-glycerophosphate.

2. On the acid side of pH optima the rate of hydrolysis with dilute substrate was greater than with substrate present in high concentrations.

3. At relatively high pH values, hydroxyl ions inhibited the hydrolysis of dilute glycerophosphate in a manner which suggested competitive inhibition. When velocities were measured at pH 9·88 for all dilutions of glycerophosphate, K_m decreased with increases in the amount of enzyme in the digest, i.e. K_m varied with variations in the enzyme:inhibitor ratio. Under similar conditions of pH, K_m for plasma of rachitic chicks, having a high phosphatase activity, was lower than K_m for the plasma of chicks fed with vitamin D, possessing low phosphatase activity.

4. When velocities were measured at the optimum pH for each concentration of substrate, variations in K_m due to differences in enzyme concentration were greatly reduced. The $1/v:1/[S]$ plots of velocities obtained in this manner also resolved the data into two parts from which two values for K_m were calculated for each preparation of enzyme, namely K_{m_1} for data with dilute substrate and K_{m_2} for data with high concentrations of substrate.

5. The presence or absence of vitamin D in the diet of chicks had no effect on K_{m_1} and K_{m_2} for plasma phosphatase when the velocities were measured at the optimum pH for each concentration of substrate.

6. When velocities were measured at the same pH for all concentrations of phenyl phosphate, K_m values for fowl plasma phosphatase increased with increasing alkalinity from 0·15 mM at pH 8·9 to 8·3 mM at pH 10·3.

7. K_m values, plotted as pK_m versus pH, fell along a straight line with a slope of $-1{\cdot}3$ when the velocities were measured in the presence of opti-

mum amounts of magnesium chloride in the digest. The effect of activators on the relationship between pH and K_m is discussed.

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Studies on Alkaline Phosphatases

2. FACTORS INFLUENCING pH OPTIMA AND MICHAELIS CONSTANT

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It has become quite evident that the pH required for optimum activity of alkaline phosphatases, as well as for some other enzymes, is not a constant entity. For example, the optimum pH for phosphatases from various sources has been shown to vary with the initial concentration of substrate (Motzok & Wynne, 1950; Ross, Ely & Archer, 1951; Morton, 1957). This phenomenon has been observed with other enzymes, namely urease (Van Slyke & Cullen, 1914) and β-glucosidase (Hofstee, 1955). Other factors, such as buffers (Aebi & Abelin, 1948; Zittle & Della Monica, 1950), type of substrate (Delory & King, 1943; Walker & King, 1950), source of enzyme (Zittle & Della Monica, 1950; Motzok, 1950; Morton, 1955) and activators (Motzok, 1945; Aebi & Abelin, 1948; Sadasivan, 1952; Morton, 1957) also have been found to exert an influence on the optimum pH for phosphatase activity. In our studies on the kinetics of alkaline phosphatases of tissues and plasmas of different species of birds and mammals, the pH optima were determined for a wide range of concentration of substrate with each enzyme preparation. Several factors were found to influence the optimum pH and some of the data are recorded in this paper.

It was demonstrated (Motzok, 1959) that K_m values (Michaelis constant) for chick plasma phosphatase varied with the amount of enzyme in the reaction mixture when the activities were measured at a constant high pH for all dilutions of substrate and enzyme. However, when the activities were measured at the optimum pH for each concentration of substrate, the variation in K_m values due to variation in the concentration of enzyme was largely eliminated. In addition, the plotting of reciprocals of velocities and substrate concentrations, according to Lineweaver & Burk (1934), resolved the data into two parts from which two values for K_m were calculated, K_{m_1} from the data with low concentration of substrate and K_{m_2} from

velocities with high concentrations of substrate. In the present study values for K_{m_1} and K_{m_2}, derived from velocities at the optimum pH for each concentration of substrate, were determined for the alkaline phosphatases of various tissues from different species of birds and mammals. These constants are compared with K_m values determined at a constant pH, the optimum for a concentration of substrate large enough to maintain zero-order rate of reaction.

METHODS AND MATERIALS

Methods for the preparation of plasma and tissue phosphatases and for the determination of phosphatase activity have been described previously (Motzok & Wynne, 1950; Motzok, 1950, 1959). With the exception of the study on pH optima without added Mg^{2+} ions (Fig. 3), phosphatase activities were determined in the presence of optimum amounts of $MgCl_2$ in the reaction mixtures, i.e. 5 mM for the phosphatases of plasma, bone, liver and kidney, and 0·01 M for the enzyme of intestinal mucosa. The strains and breeds used in this study were Sprague-Dawley ats, giant white rabbits, Yorkshire pigs, Chinese × Emrden goslings and Columbian Rock, Barred Plymouth Rock and New Hampshire fowls. The pigeons were of the common breeds. The goslings were raised on a practical type of diet (Branion & Hill, 1952). The other birds and the mammals were fed with commercial diets.

EXPERIMENTAL AND RESULTS

Influence of species of birds and mammals on pH *optima of intestinal phosphatase*

Fig. 1 shows that the phosphatase of intestinal mucosa of rats exhibited optimum activity with each concentration of β-glycerophosphate at the lowest pH values and the enzyme from the intestinal mucosa of swine had optimum activities at the highest pH values. There was little difference in the pH optima for the enzymes of goslings and of rats, on the one hand, and in the pH optima for the enzymes of pigeons and of swine, on the other. The pH optima for the enzyme of rabbits were somewhat lower than those for the enzyme of swine. In the avian class the intestinal enzyme of fowls (Columbian Rock) exhibited maximal activities at pH values which were intermediate between those required by the enzyme of goslings and those needed by the enzyme of pigeons.

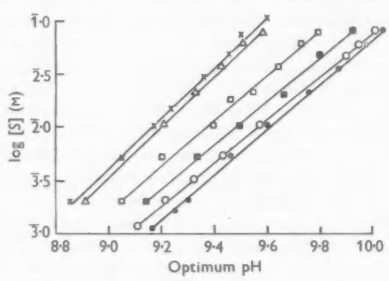

Fig. 1. Relationship between initial concentration of sodium β-glycerophosphate, expressed logarithmically, and pH for optimum alkaline phosphatase activity of intestinal mucosa of rats (×), goslings (△), fowls (□), rabbits (■), pigeons (○) and pigs (●). Activities were measured in the presence of 0·01 M-$MgCl_2$.

The data also show that a linear relationship exists between the logarithm of the initial concentration of substrate and the respective optimum pH for each enzyme preparation, the shift being to higher pH values with increasing concentrations of substrate. It was also found that the linear relationship between substrate concentrations, expressed logarithmically, and pH optima for the phosphatase of rat intestinal mucosa extended to physiological pH values with dilutions of substrate beyond the amounts shown in Fig. 1. With an initial concentration of 0·02 mM-substrate the optimum was pH 7·7. This confirms the findings of Ross *et al.* (1951) that with very dilute substrate the optimum pH for alkaline phosphatase is near neutral pH values.

pH *optima of phosphatases of different tissues*

Rat. Fig. 2 (*A*) shows that the pH optima for the enzymes of bone and kidney of rats were practically the same for each concentration of β-glycerophosphate, whereas the optima for the enzyme of liver were slightly lower. The differences in the pH optima for the phosphatases of plasma and intestinal mucosa were very small, if not negligible. These optima, however, were considerably below those for the enzymes of bone and kidney.

Rabbit. The phosphatases of liver and kidney of rabbits (Fig. 2*B*) were found to possess the highest pH optima with β-glycerophosphate recorded in these studies. In fact, the pH optima for high concentrations of substrate could not be determined because these appeared to be beyond the effective range of the buffer system employed. The pH optima for the phosphatases of bone and intestinal mucosa were considerably lower than those for the enzymes of liver and kidney, whereas plasma phosphatase exhibited maximum activities at intermediate values of pH.

Pigeon. The phosphatases of bone, kidney and intestinal mucosa of pigeons (Fig. 2*C*) appeared to possess comparable pH optima for the respective concentrations of β-glycerophosphate. On the other hand, the pH optima for the enzyme of liver were considerably lower than those required by the enzymes of the other tissues.

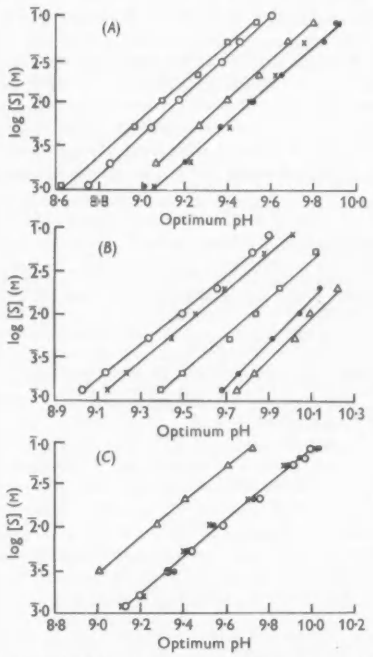

Fig. 2. Relationship between initial concentration of sodium β-glycerophosphate, expressed logarithmically, and pH for optimum alkaline phosphatase activity of plasma (□), intestinal mucosa (○), liver (△), kidney (●) and bone (×). (*A*) Rats; (*B*) rabbits; (*C*) pigeons. Activities were measured in the presence of optimum concentrations of $MgCl_2$ in the reaction mixtures: 5 mM for the phosphatases of plasma, bone liver and kidney and 0·01 M for the phosphatase of intestinal mucosa.

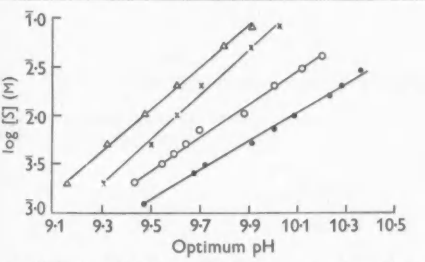

Fig. 3. Relationship between initial concentrations of substrate, expressed logarithmically, and the pH for optimum activities of alkaline phosphatase of intestinal mucosa of fowls (sodium β-glycerophosphate as substrate) in the presence of 0·01 M-$MgCl_2$ (△) and without added magnesium (×), and of plasma phosphatase of fowls (disodium phenyl phosphate as substrate) in the presence of 5 mM-$MgCl_2$ (●) and without added magnesium (○).

Influence of magnesium on pH *optima*

The data in Fig. 3 show the influence of added $MgCl_2$ on the pH optima required by alkaline phosphatase of plasma of fowls with phenyl phosphate as the substrate and by the enzyme of intestinal mucosa with sodium β-glycerophosphate. A linear relationship between the initial concentrations of substrate, expressed logarithmically, and the pH optima existed with and without added magnesium. However, the addition of magnesium to the reaction mixture changed the pH optima for both plasma and intestinal mucosa phosphatases, causing the optima to shift to higher values for the enzyme of plasma and to lower values for the enzyme of intestinal mucosa.

K_m values at optimum pH *for each concentration of substrate*

In studies on the kinetics of alkaline phosphatases of plasma and tissues of several species of birds and mammals, pH optima were determined for a wide range of concentration of substrate with each enzyme preparation. The activities of the phosphatases from various sources were measured at the optimum pH for each concentration of substrate and the velocities were plotted according to the method of Lineweaver & Burk (1934). The $1/v:1/[S]$ relationship for all enzyme preparations was similar to those found previously (Motzok, 1959, figs. 4 and 8). The points which fell along straight lines were used for the calculation of the lines of best fit (Treloar, 1939). The constants, K_{m_1} for dilute substrate and K_{m_2} for substrate present in high concentrations, were derived from the calculations of the lines of best fit and are presented in Tables 1 and 2.

Twelve preparations of plasma phosphatase and six preparations of the enzyme of intestinal mucosa were obtained from several breeds of chicks used in diverse experiments and varying in age from 3 to 8 weeks. The average values for K_{m_1} and K_{m_2} (Table 1) were 2·1 mM and 22·5 mM for plasma phosphatase and 2·2 mM and 9·0 mM for the intestinal enzyme. Whereas the values for K_{m_1} for the phosphatases of the two sources were quite similar, the average values for K_{m_2} for plasma phosphatase were more than twice that for the enzyme of intestinal mucosa. Although K_{m_1} values for intestinal phosphatase of mature fowls were somewhat lower than those for the enzyme of chicks, the values for K_{m_2} were within the range of values found for the young birds. The data for chicks and mature birds were arranged in increasing order on the numerical values for K_{m_1} to show that variations in the respective values for K_{m_2} were not related to the variations in K_{m_1}.

Values for K_{m_1} for plasma phosphatases of goslings and chicks were about the same, whereas K_{m_2} for the plasma enzyme of goslings was only about one-tenth of K_{m_2} for the enzyme of chicks. Values for K_{m_1} and K_{m_2} for intestinal phosphatase of pigs and calves were comparable with those for fowls. The calf intestinal phosphatase was a commercial product, prepared by partial purification according to the method of Schmidt & Thannhauser (1943).

With disodium phenyl phosphate as the substrate for intestinal phosphatase of chicks, the values for K_{m_1} and K_{m_2} were lower than the corresponding constants obtained with β-glycerophosphate. With calf intestinal phosphatase, the value of 2 mM for K_{m_1} for phenyl phosphate was

Table 1. *K_m values of alkaline phosphatases from various sources*

Activities were determined at the respective pH optima for the different concentrations of substrate with each enzyme preparation. K_{m_1} and K_{m_2} values were calculated from activities with low and high concentrations of substrate respectively and plotted according to Lineweaver & Burk (1934).

	K_m (mM)					
	Sodium β-glycerophosphate				Disodium phenyl phosphate	
	Plasma		Intestinal mucosa		Intestinal mucosa	
Species	K_{m_1}	K_{m_2}	K_{m_1}	K_{m_2}	K_{m_1}	K_{m_2}
Chick	1·8	23	1·8	9	0·8	2·3
	1·9	15	1·9	9	0·9	2·3
	1·9	15	2·3	8	1·2	3·8
	1·9	17	2·3	12	1·3	3·2
	2·1	30	2·4	11		
	2·2	20	2·5	7		
	2·2	20				
	2·2	23				
	2·2	26				
	2·2	30				
	2·2	30				
	2·4	21				
Mature fowl	—	—	1·3	7	—	—
			1·5	6		
			1·5	6		
Goose	1·5	2·7	2·4	11	—	—
Pig	—	—	2·1	10	—	—
Calf*	—	—	1·7	11	2·2	†

* Calf intestinal mucosa purified commercially by the method of Schmidt & Thannhauser (1943).

† No estimate was possible because pH optima for high concentrations of phenyl phosphate were beyond the effective range of the buffer system.

Table 2. *K_m values of alkaline phosphatases of rat, rabbit, and pigeon*

Activities were determined at the respective pH optima for the different concentrations of sodium β-glycerophosphate with each enzyme preparation. K_{m_1} and K_{m_2} values were calculated from activities with low and high concentrations of substrate respectively and plotted according to Lineweaver & Burk (1934).

	K_m (mM)					
	Rat		Rabbit		Pigeon	
Source of enzyme	K_{m_1}	K_{m_2}	K_{m_1}	K_{m_2}	K_{m_1}	K_{m_2}
Plasma	1·3	16	*	*	*	*
Intestinal mucosa	1·6	9	1·2	5	2·4	11
Bone	1·6	11	1·9	11	1·9	10
Kidney	1·3	11	2·1	†	3·0	11
Liver	‡	‡	2·0	†	2·1	19

* No estimate was possible owing to low phosphatase activity.

† No estimate was possible because pH optima for high concentrations of substrate were beyond the effective range of the buffer system.

‡ No estimate was possible owing to high content of inorganic phosphate in the enzyme preparation.

comparable with the value obtained with β-glycerophosphate for the intestinal enzyme of other species. The pH optima for calf intestinal phosphatase were considerably higher with phenyl phosphate than with β-glycerophosphate (unpublished data) and activities with high concentrations of phenyl phosphate could not be determined because the pH optima appeared to be beyond the range of the buffer system employed.

In Table 2 are shown the values for K_{m_1} and K_{m_2} for the phosphatases of several sources in rats, rabbits and pigeons with β-glycerophosphate as the substrate. It appears that K_{m_1} values for intestinal mucosa and kidney phosphatases of pigeons were somewhat higher than those for the enzymes of the same sources in rats and rabbits, whereas values for K_{m_1} and K_{m_2} for bone phosphatase were quite similar in the three species.

The preparation of rat liver was found to contain an appreciable amount of inorganic phosphorus. This reduced the sensitivity of the method for determining the inorganic phosphate liberated enzymically and K_m values were not calculated. It was also observed that the phosphatase activity of the plasmas of pigeons and rabbits was very low and the method employed was inadequate to measure accurately differences in reaction velocities.

K_m values at a constant pH *for all concentrations of substrate*

K_m values for a number of preparations of phosphatase from several sources were also calculated from velocities measured at a constant pH for all concentrations of substrate. In each case the pH used was optimum for a concentration of substrate large enough to maintain zero-order rate of reaction. This study was made on the same preparations of enzymes used in obtaining the data in Tables 1 and 2 and the values presented in Table 3 can be compared directly with the data on the corresponding preparations recorded in Tables 1 and 2.

These studies show that, when the activities are measured at a constant pH for all concentrations of substrate, there is considerable variation in the calculated values for K_m for the phosphatases of various sources. In addition, these K_m values are in some cases four and five times the corresponding values for K_{m_1} calculated from velocities at the optimum pH for each concentration of substrate.

DISCUSSION

Factors affecting pH *optima*

The direct linear relationship between substrate concentration, expressed logarithmically, and optimum pH, reported by Ross *et al.* (1951) for the alkaline phosphatase of intestinal mucosa of rats, is exhibited by the alkaline phosphatases of various tissues of a number of species of birds and mammals. There are, however, marked differences in the pH optima for the enzyme of a specific tissue from different sources, e.g. intestinal phosphatase (Fig. 1), which cannot be related to differences in families, orders or even classes of vertebrates. In addition, there appears to be no explanation for some of the differences and similarities in pH optima for the phosphatases of various tissues in the same animal. For example, in pigeons (Fig. 2*C*) the optimum pH with each concentration of substrate was the same for the phosphatases of bone,

Table 3. *K_m values of alkaline phosphatases of various sources*

Activities were determined at one pH for all concentrations of substrate with each preparation of enzyme.

Substrate	Species	Source	pH*	K_m (mM)†
Sodium β-glycerophosphate	Mature fowl	Intestinal mucosa	9·7	7·6
		Intestinal mucosa	9·7	8·3
		Intestinal mucosa	9·7	10·9
	Pigeon	Intestinal mucosa	9·95	6·1
		Bone	9·95	11·0
		Kidney	10·0	14·8
		Liver	9·8	5·1
	Rabbit	Intestinal mucosa	9·9	2·6
		Bone	9·98	10·7
		Kidney	10·1	6·7
		Liver	10·16	5·4
	Calf	Intestinal mucosa	9·9	11·5
	Pig	Intestinal mucosa	9·95	8·8
Disodium phenyl phosphate	Chick	Intestinal mucosa	10·0	3·1
	Calf	Intestinal mucosa‡	10·0	4·0

* Optimum pH for a concentration of substrate large enough for zero-order reaction rate.
† Calculated from activities plotted according to Lineweaver & Burk (1934).
‡ Calf intestinal mucosa purified commercially by the method of Schmidt & Thannhauser (1943).

kidney and intestinal mucosa, whereas in rats (Fig. 2*A*) the pH optima were the same for the phosphatases of bone and kidney and markedly different for the enzyme of intestinal mucosa. In the chick it was found (Motzok, 1950) that the pH optima were the same for the phosphatases of bone, kidney, liver and plasma, whereas the optimum pH for the enzyme of intestinal mucosa was considerably lower. In the present study the data show that in rats the pH optima for the enzymes of plasma and intestinal mucosa are quite similar (Fig. 2*A*).

Bodansky (1934) concluded that the source of phosphatase in blood was of diverse origin. In diseases of liver and bone he considered the increase in serum phosphatase to arise from the particular organ that was involved. Kay (1933) had suggested that the phosphatase of serum was identical with that of various tissues, being the result of leakage into the circulatory system from tissues of higher phosphatase content. Motzok (1950) presented evidence that in rachitic chicks the skeleton is the main source of the increased plasma phosphatase. The similarity in the pH optima for the phosphatases of plasma and intestinal mucosa in rats (Fig. 2*A*) lends support to the suggestion of Flock & Bollman (1948) and Madsen & Tuba (1951) that in the rat the alkaline phosphatase is supplied to the plasma by the small intestine.

If the phosphatase of plasma in rats originates in the intestinal mucosa, the similarity in pH optima for the enzymes of the two sources suggests that the environmental medium of the plasma did not contain factors which altered the characteristics of the enzyme governing the pH required for optimum activity *in vitro* under the conditions of the experiment. The data in Fig. 3, however, show that the addition of magnesium may exert an opposite effect on pH optima for phosphatase of different sources, e.g. raising the pH optima for the phosphatase of plasma and lowering the pH optima for the enzyme of intestinal mucosa of fowls. It was shown previously (Motzok, 1945) that the addition of magnesium also caused the pH optima for plasma phosphatase of chicks to shift to higher values with β-glycerophosphate. Therefore the shift in pH optima in opposite directions for intestinal phosphatase with β-glycerophosphate and plasma phosphatase with phenyl phosphate cannot be attributed to the type of substrate used in measuring phosphatase activity. The addition of magnesium does not change the pH optima of alkaline phosphatase of all sources, as was shown by Aebi & Abelin (1948), who found that added magnesium had no effect whereas added manganese caused a definite shift in optimum pH for the unpurified enzyme of rat kidney. It appears that the factors which determine the optimum pH of a particular phosphatase reside in the nature of the enzyme. Consequently, the manner and degree of the influence of environmental factors are probably governed by the characteristics of the enzyme itself. There remains for further study the effect of activators on the pH optima for phosphatases of other sources.

The present findings serve to emphasize the fallacy of applying optimum conditions established for the phosphatase of one source to the phosphatases of other sources. Consideration of all known factors is desirable in order to avoid further confusion in the literature with respect to pH optima of alkaline phosphatase, as indeed of other enzymes.

Factors influencing K_m values

The problem of the choice of pH for measuring phosphatase activity with a range of concentration of substrate large enough for an accurate estimation of K_m has been discussed (Motzok, 1959). The data in Table 3 show that there was considerable variation in K_m derived from activities measured at a constant pH for all concentrations of substrate. At least a part of the variation in K_m derived in this manner was found to be caused by variation in the amount of enzyme in the reaction mixture (Motzok, 1959). Even if the amount of enzyme employed were kept constant on the basis of activity, one cannot safely assume that the pH–activity curves with each concentration of substrate are the same for the phosphatases from all sources. Certainly, the use of one pH for all phosphatases would yield K_m values which would be affected to an appreciable extent by differences in pH optima which exist among species and tissues of individual species. The influence of other factors, such as buffers and activators, on pH optima would also have to be taken into consideration.

Van Slyke (1942) has pointed out that, although K_m is a useful value, it can be called a constant only for defined conditions; e.g. a drop of 1 unit in pH will increase the K_m of urease 11-fold (Van Slyke & Cullen, 1914). Similarly, K_m values for phosphatases, which have been recorded in the literature, can at best be regarded as being applicable only to the particular preparation of enzyme used and for the specific set of conditions *in vitro* under which the activities were measured. Unfortunately, in many instances no details of the methods employed have been recorded. In cases where such information has been given, the direct comparison of K_m values, as distinguishing characteristics of phosphatases, could be made only with due regard to various factors such as those mentioned above.

It was shown previously (Motzok, 1959) that the effect of the amount of enzyme in the reaction

mixture on K_m, when the velocities were measured at a constant pH, was largely eliminated by measuring velocities at the optimum pH for each concentration of substrate. Indeed, there was only a small variation in the values for K_{m_1} and K_{m_2} for the phosphatases of a number of preparations of plasma and intestinal mucosa of chicks (Table 1) when the optimum pH for each concentration of substrate was used. Considerably more data are required on the phosphatases of other sources with different substrates to evaluate fully the differences and similarities in the values for K_{m_1} and K_{m_2} reported in this paper.

SUMMARY

1. Marked differences, and in some cases unexpected similarities, were found in the pH optima (for the respective concentrations of substrate) required for the activities of the alkaline phosphatases from various tissues of different species of birds and mammals.

2. Linear relationships were shown to exist between the initial concentrations of substrate, expressed logarithmically, and the pH optima for the activities of the phosphatases from various sources. The optimum pH shifted to higher values with increasing concentration of substrate.

3. The addition of magnesium concentrations (5 mM for plasma phosphatase and 0·01 M for intestinal phosphatase) for maximum activation caused the respective pH optima to shift to higher values for the plasma phosphatase and to lower values for the intestinal enzyme of fowls when compared with pH optima without the addition of magnesium.

4. By the use of velocities at the optimum pH for each concentration of substrate, values for K_{m_1} from data with dilute substrate, and K_{m_2} from data with high concentrations of substrate, were calculated for the phosphatases of various tissues and plasma of fowls, geese, pigeons, rats, rabbits, pigs and calves.

5. The average values for K_{m_1} and K_{m_2} with β-glycerophosphate were 2·1 mM and 22·5 mM for 12 preparations of plasma, and 2·2 mM and 9 mM for six preparations of intestinal mucosa of chicks. With phenyl phosphate, the average values for K_{m_1} and K_{m_2} for four preparations of intestinal mucosa of chicks were 1 and 3 mM. Variations in the values for K_{m_1} and K_{m_2} were quite small. More data are required on the phosphatases of other sources with different substrates to evaluate the similarities and differences in the constants recorded.

6. There was considerable variation in K_m derived from velocities measured at a constant pH for all concentrations of substrate.

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The Component Fatty Acids of *Penicillium spinulosum* Fat

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The fatty acid ingredients in fat synthesis in *Aspergillus nidulans* and in *Penicillium lilacinum* have been identified and estimated by Singh, Walker & Meara (1955) and by Singh, Shah & Walker (1956*a*). *Penicillium spinulosum* produces fat in high yield when grown in a chemically defined glucose medium (Garrido & Walker, 1953; Garrido, Woodbine & Walker, 1953) or in a similar medium in which sucrose is the source of carbon (Gad & Walker, 1954), and, judging from the results of a preliminary nutritional trial, this fat does not seem to be toxic to rats (personal communication from Dr T. Moore, Dunn Nutritional Laboratory, Cambridge). More extensive trials of this fat as food are planned and its fatty acid composition has therefore been determined.

EXPERIMENTAL

The strain of *P. spinulosum* was isolated in this Laboratory and its identity was confirmed at the Centraalbureau voor Schimmelcultures, Baarn, Holland (personal communication). It was grown in a medium which contained (g./l.): $NaH_2PO_4,2H_2O$, 0·270; $MgSO_4,7H_2O$, 0·090; K_2SO_4, 0·082; $ZnSO_4,7H_2O$, 0·013; $FeCl_3,6H_2O$, 0·040; NH_4Cl, 3·20; glucose, 100. These constituents were made up with tap water and the pH was adjusted with NaOH to 6·8. This medium (20 l.) was sterilized in steam at 10 lb./in.^{2} for 15 min., cooled and introduced aseptically into a stainless-steel fermentation vessel which had already been sterilized by a current of steam. This vessel was fitted with an impeller geared to revolve at 500 rev./min. After inoculation of the medium with a suspension of spores, stirring was commenced and a stream of air (10 l./min.) was led through the medium for 7 days at 27°. The contents of the vessel were then drawn off and the whole (mycelium and metabolism solution) was sterilized at 100° for 10 min. The mycelial tissue was separated by filtration, washed with hot water, pressed, spread on filter paper, dried for 2 days in a current of air at 27° and dried finally for 3 hr. in an oven at 60°. The material was then ground in a large coffee-mill, placed in a Soxhlet apparatus and extracted with light petroleum (b.p. 40–60°). After removal of the solvent, 125 g. of fat was obtained from 300 g. of dried felt. The fat was a reddish yellow liquid which, on examination according to British Standard Methods of Analysis of Oils and Fats (1950; B.S. 684), gave the following figures: saponification equivalent, 294·2; iodine value, 81·1; free fatty acids (as oleic acid), 5·8%; unsaponifiable matter, 1·4%. The greater part (120 g.) of the fat was saponified with 750 ml. of ethanol containing 6% (w/v) of KOH.

After removal of the bulk of the ethanol by distillation, the soaps were taken up in water and unsaponifiable matter was removed by ether. To the aqueous solution (600 ml.), 250 ml. of 4 N-H_2SO_4 was added and the liberated component acids were taken up in ether, washed, dried (104·91 g.) and subjected to low-temperature crystallization by the procedure of Hilditch (1956). A solution (10%, w/v) of the mixed fatty acids in acetone was cooled in a bath of ethanol–solid CO_2 to −72° and maintained at that temperature until crystals separated. After 6 hr. these were filtered off with continued strong cooling, the compounds remaining in solution at this temperature constituting group D. The separated crystals were then recrystallized from solution (10%, w/v) in acetone by cooling to −40°. This afforded a soluble group (C), and an insoluble group which was recrystallized from solution (10%, w/v) in ether by cooling to −25°, whereby a soluble group (B) was separated from an insoluble group (A) (Table 1). Spectrophotometric determinations on groups A, B, C and D isomerized under standard conditions (Hilditch, Riley & Patel, 1951) after removal of unsaponifiable matter afforded the further data shown in Table 1.

Table 1. *Fractionation of mixed fatty acids of* Penicillium spinulosum *fat by low-temperature crystallization*

For explanation of groups A, B, C and D, see text.

Group	Conditions	Wt. (g.)	Percentage (w/w)	Iodine value	$E^{1\%}_{1\,cm.}$ (234 mμ)*	$E^{1\%}_{1\,cm.}$ (268 mμ)
A	Insoluble in 10 vol. of acetone at −40° and in 10 vol. of ether at −25°	29·50	28·1	7·9	—	—
B	Insoluble in 10 vol. of acetone at −40°, soluble in 10 vol. of ether at −25°	22·60	21·5	72·6	14·5	—
C	Soluble in 10 vol. of acetone at −40°	26·93	25·7	101·5	151	—
D	Soluble in 10 vol. of acetone at −72°	25·88	24·7	154·6	641	6

* After isomerization with alkali.

Table 2. *Fractionation of methyl esters of acids of groups A, B, C and D*

Fraction	Wt. (g.)	Saponification equivalent	Iodine value
	Methyl esters of acids A		
A_1	0·93	271·8	2·2
A_2	1·42	275·5	2·7
A_3	1·96	275·8	3·8
A_4	1·65	277·7	2·4
A_5	1·97	278·7	2·7
A_6	1·86	279·5	4·9
A_7	1·71	281·1	8·7
A_8	1·90	291·2	9·6
A_9	2·49	291·5	10·6
A_{10}	1·06	299·5	11·6
A_{11}	1·85	303·4	11·9
A_{12}	1·87	305·0	13·5
	Methyl esters of acids B		
B_1	1·66	273·5	4·6
B_2	1·93	286·6	63·4
B_3	1·59	284·4	67·6
B_4	1·73	289·5	71·3
B_5	1·57	290·1	72·1
B_6	1·73	292·2	73·7
B_7	1·27	293·7	76·5
B_8	1·81	295·5	77·6
B_9	1·40	297·5	77·0
	Methyl esters of acids C		
C_1	1·82	289·3	91·3
C_2	2·10	292·5	94·7
C_3	2·03	292·3	97·5
C_4	2·04	295·7	99·2
C_5	2·22	295·8	98·2
C_6	2·10	295·9	99·3
C_7	3·34	298·0	99·0
C_8	2·03	301·1	98·0
C_9	1·40	301·1	98·0
	Methyl esters of acids D		
D_1	1·17	272·0	120·0
D_2	2·27	288·0	132·4
D_3	1·66	293·0	150·6
D_4	2·67	293·7	152·3
D_5	3·29	293·5	153·0
D_6	2·18	294·9	152·8
D_7	3·74	295·2	152·0
D_8	0·98	311·0	141·1

Determination of component fatty acids

The different groups A, B, C and D were esterified separately (Bjarnason & Meara, 1944) and fractionally distilled under reduced pressure (0·1 mm. Hg) through an electrically heated column of the type described by Longenecker (1937). By taking suitably small fractions of the distillate it was possible to obtain not more than two homologous saturated acids and two unsaturated acids of the same series in each fraction. Table 2 shows the saponification equivalent and iodine value of each fraction. From the analytical data obtained for each ester fraction and from the appropriate spectroscopic data, its composition and thence that of each acid group and ultimately that of the whole fat was then ascertained (Table 3) in accordance with the general principles which Hilditch (1956) enunciated.

Identification of fatty acids

Specimens of free acids regenerated from fractions A_1–A_5 were recrystallized from ethanol, when each lot of crystals had m.p. 61·7–63·0° alone or when mixed with pure palmitic acid. From the saturated acids liberated from fractions A_8–A_{11}, after oxidation according to Lapworth & Mottram (1925), an acid was obtained of m.p. 69·3–69·9° undepressed by admixture with stearic acid of m.p. 70°. The presence of oleic acid in acids recovered from fractions B_8–B_9 and C_1–C_3 was established by conversion into 9:10-dihydroxystearic acid which, after crystallization from hot ethyl acetate, melted at 129·3–131·5°, alone or in admixture with authentic 9:10-dihydroxystearic acid of m.p. 131°. Fractions D_3–D_7 afforded linoleic acid, which was characterized by bromination in light petroleum (b.p. 40–60°), whereby it yielded tetrabromostearic acid, m.p. 113·2°, alone or when mixed with an authentic specimen of m.p. 114°.

DISCUSSION

The relatively high content of free fatty acids often noted in the fats of micro-organisms, particularly yeasts, would be a drawback if it were desired to incorporate such fats in feeding-stuffs. *P. spinulosum* fat has a higher free fatty acid content (5·8 % as oleic acid) than any of the fats of five other moulds studied in these Laboratories

Table 3. *Component fatty acids of* Penicillium spinulosum *fat*

	Acid groups				Total fatty acids, excluding unsaponifiable material (%, w/w)
	A (28·1 %)* (%)†	B (21·5 %)* (%)†	C (25·7 %)* (%)†	D (24·7 %)* (%)†	
Palmitic acid	46·3	19·5	2·3	1·2	18·0
Stearic acid	40·9	1·8	0·4	—	11·9
Arachidic acid	3·9	0·5	0·8	—	1·4
Hexadecenoic acid	—	7·4	3·5	4·9	3·8
Oleic acid	8·9	69·4	76·6	24·7	43·3
Linoleic acid	—	1·4	16·3	67·1	21·1
Linolenic acid	—	—	—	1·2	0·3
Eicosenoic acid	—	—	—	0·8	0·2
Unsaponifiable matter	—	—	0·1	0·1	—

* Groups as % (w/w) of total acids.

† Component acids as % (w/w) of group.

(Singh *et al.* 1955; Singh *et al.* 1956*a*; Singh, Shah & Walker, 1956*b*; Singh, 1957; Singh, Philip & Walker, 1957). On the other hand, even this value is much lower than the corresponding values reported for yeast fats. For example, the free acidity of *Rhodotorula gracilis* fat is 18 % (Holmberg, 1948) and that of the fat of yeast strain no. 72 is 33 % (Hilditch & Shrivastava, 1948).

Table 4 permits comparisons of the analytical data for the fat of *P. spinulosum* with the figures for the fats of *A. nidulans* and *P. lilacinum*. Data derived from analysis of palm oil (Bank, Dean & Hilditch, 1935), olive oil (Gunde & Hilditch, 1940) and groundnut oil (Crawford & Hilditch, 1950) are also shown for comparison with the figures relating to the mould fats, because one of the objects of the present work was to find whether mould fats could be used in special cases as substitutes for vegetable oils.

Singh *et al.* (1955) drew attention to the fact that, in the triglyceride portion of fats of microbiological origin reported up to that time, 'the palmitic acid content, while being somewhat variable, constitutes about 70 % of the saturated acids present, the remaining acids consisting of stearic acid and smaller amounts of myristic, and the higher arachidic, behenic and lignoceric acids'. Further, these authors proceeded to show that in *A. nidulans* fat the palmitic acid content was only 5 units % higher than the stearic acid content, palmitic acid constituting 52·7 % of the total saturated fatty acids. A similar quantitative relationship between the palmitic acid and stearic acid components of a fat is now seen in *P. spinulosum* fat, for the quantity of palmitic acid incorporated into it is only 6·1 units % higher than the quantity of stearic acid combined in this fat; the palmitic acid component constitutes 57·7 % of the total saturated fatty acids. Accordingly, *P. spinulosum* fat affords another example of a mould fat which resembles to some extent the seed fats of certain tropical plants, e.g. *Madhuca latifolia* (Hilditch & Ichaporia, 1938). Whereas stearic acid thus constitutes more than one-third of the total saturated acids which are combined in the glycerides of *P. spinulosum* fat, the proportions in which this acid occurs in the three vegetable oils listed in Table 4 are much lower.

The content of combined hexadecenoic acid in *P. spinulosum* fat is low (3·7 %) in comparison with the amounts (15–20 %) of this acid in marine-animal oils, but the percentage is high in comparison with the values (usually less than 1 % of the total fatty acids) found for this acid in cottonseed oil, peanut oil, soya-bean oil and palmo il. A low percentage of hexadecenoic acid seems to be a characteristic of the fats of a number of moulds which are capable of synthesizing large amounts of

Table 4. *Component fatty acids of* Penicillium spinulosum *fat compared with the component fatty acids of fats from other moulds and from vegetable oils*

	Free acidity (as oleic acid %)	Iodine value	Saturated acids				Unsaturated acids				
			C_{14}	C_{16}	C_{18}	C_{20-24}	C_{16} ($-2H$)	C_{18} ($-2H$)	C_{18} ($-4H$)	C_{18} ($-6H$)	C_{20} ($-2H$)
Penicillium spinulosum	5·8	81·1	Trace	18·0	11·9	1·4	3·8	43·3	21·1	0·3	0·2
Aspergillus nidulans	0·8	73·4	0·7	20·9	15·9	1·4	1·2	40·3	17·0	0·2	2·4
Penicillium lilacinum	0·2	63·7	0·1	32·3	9·4	1·4	3·4	38·6	13·4	Nil	1·4
Palm oil*	Not given	60·0	1·6	32·3	5·5	Nil	Nil	52·4	8·2	Nil	Nil
Olive oil†	Not given	84·2	1·2	15·6	2·0	Nil	1·6	64·6	15·0	Nil	Nil
Groundnut oil‡	Not given	86·5	Nil	8·8	4·5	6·1	Nil	60·3	20·3	Nil	Nil

* Results of Bank *et al.* 1935. † Results of Gunde & Hilditch, 1940. ‡ Results of Crawford & Hilditch, 1950.

fat from carbohydrate. For example, the figures for hexadecenoic acid in the fats of *A. nidulans* and *P. lilacinum* are respectively 1·2 and 3·4 % (Table 4); likewise the corresponding figures for the fats synthesized by *Penicillium flavo-cinereum* and *Penicillium soppi* are 1·5 and 3·3 % respectively (Singh *et al.* 1956*b*; Singh *et al.* 1957). Thus the proportions of combined hexadecenoic acid in the fats of these particular moulds are fairly close to those found in mammalian depot fats (2–3 %) and milk fats (3–4 %) (Hilditch, 1956).

In *P. spinulosum* fat the proportion of combined oleic acid, although of much the same order as in the fats of certain other moulds (*A. nidulans*, *P. lilacinum*, *P. flavo-cinereum* and *P. soppi*), is substantially less than in palm oil, olive oil or groundnut oil (Table 4).

Finally, in *P. spinulosum* fat the proportion (21·1 %) of combined linolenic acid lies between the proportions in which it occurs in the fats of *A. nidulans* (17·0 %) and of *A. flavus* (23·7 %) (Singh, 1957). The corresponding value for *P. soppi* fat was found by Singh *et al.* (1957) to be 20·0 %. The lowest and highest values for this component in mould fats examined by Singh *et al.* (1956*a*, *b*) are *P. lilacinum* (13·4 %), *P. flavo-cinereum* (27·1 %). Whereas the linoleic acid content of groundnut oil may vary, in different samples, from a value (20 %) very close to that of *P. spinulosum* fat to a much higher figure (35 %), neither palm oil (8·2 %) nor olive oil (15·0 %) incorporates so high a proportion of this unsaturated acid (Table 4). In view of the importance of linoleic acid in nutrition, the fat of *P. spinulosum* should compare favourably, in this particular respect, with these three vegetable oils.

SUMMARY

1. The component acids of fat produced by *Penicillium spinulosum* grown in aerated deep culture have been found to be (%): palmitic, 18·0; stearic, 11·9; arachidic, 1·4; hexadecenoic, 3·8; oleic, 43·4; linoleic, 21·1; linolenic, 0·3; eicosenoic, 0·2.

2. The fat, like the fats of the moulds *Aspergillus nidulans* and *Aspergillus flavus*, has a relatively high stearic acid content in comparison with the fats of other micro-organisms.

3. The hexadecenoic acid content, like the hexadecenoic acid contents of the fats obtained from a number of other fat-rich mould fungi, is small.

4. The linoleic acid content of the fat is greater than the linoleic acid contents of the more unsaturated olive oils and approaches the proportions normally present in groundnut oils.

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Pyruvate Metabolism in *Lactobacillus brevis*

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The pyruvate metabolism of *Lactobacillus plantarum* and *Lactobacillus arabinosus*, which according to Bergey (1948) are identical organisms, has been studied by Rowatt (1951) and Nossal (1952). Both these homofermentative lactobacilli were shown to form 3-hydroxybutan-2-one (acetoin) as the main product of their pyruvate metabolism. No one appears to have studied the metabolism of pyruvate by any of the heterofermentative lactobacilli, of which *Lactobacillus brevis* is a typical example. This species frequently occurs in cheddar cheese, in the ripening of which it may play a significant part. It was considered of interest therefore to compare the pyruvate metabolism of *L. brevis* with that of the homofermentative species.

MATERIALS AND METHODS

Growth of cells. The organism used throughout these studies was *L. brevis* strain 1·2, originally isolated from New Zealand cheddar cheese by Sherwood (1939). Throughout this work stock cultures were maintained in sterile skim milk containing 0·2% of yeast extract (Oxoid, Oxo Ltd.). Cells for experimental use were grown in the lactose–yeast–phosphate broth of Hunter (1946) for 48 hr. at 30°. After centrifuging, the cells were washed twice and finally resuspended in sterile 1% (w/v) KCl. Individual cell crops showed random variation in yield and activity; a similar phenomenon was reported by Nossal (1952) with *L. arabinosus*. This fact, together with the slow growth rate of *L. brevis*, made it difficult to obtain exactly reproducible quantitative results; for example, the final yields of CO_2 sometimes fell short of the theoretical quantity by amounts between 10 and 20%. The overall qualitative picture, however, was not affected.

Reagents. Pyruvic acid and 2-oxobutyric acid were redistilled *in vacuo*. Solutions of their sodium salts were prepared by suitable dilution and neutralization (with NaOH). Lithium lactate (British Drug Houses Ltd., Poole, Dorset, England) was purified by dissolving in a minimum of hot water, filtering, precipitating with ethanol and drying over $CaCl_2$. Other compounds were commercial products.

Measurement of gas changes. Oxygen uptake and CO_2 production were measured by conventional Warburg techniques at 30°. Carbon dioxide was absorbed in KOH for O_2-uptake determinations and the values so obtained were used to correct the values for CO_2 evolution.

Each vessel contained 0·5 ml. of cell suspension and 1·5 ml. of 0·2M-phosphate–citrate buffer (McIlvaine, 1921), pH 4·8, unless otherwise stated. Except in cofactor experiments, 0·1 ml. of 0·01M-thiamine and 0·1 ml. of 0·01M-$MnSO_4$ soln. were added to all flasks. Substrate(s) was added from the side arm after equilibration. The total volume of fluid per flask was adjusted to 3 ml. in all experiments.

Analytical methods

At the conclusion of an experiment the Warburg flask contents were centrifuged to remove cells and the clear supernatants stored in the refrigerator before analysis.

Acetoin. This was estimated by a quantitative Voges–Proskauer reaction according to Westfeld (1945).

α-Acetolactic acid. This compound has been shown by Juni (1952) to be decarboxylated to acetoin in acid solutions and this method was used to test for the presence of this compound.

Diacetyl. This was assayed by the specific method of White, Krampitz & Werkman (1946).

Keto acids. These were estimated as 2:4-dinitrophenylhydrazones by the method of Friedemann & Haugen (1943).

Lactic acid. This was estimated by the method of Barker & Summerson (1941). A control tube containing 10 μmoles of pyruvate did not give significant colour development under the conditions used.

Volatile fatty acids. These were estimated by the method of McLendon (1944), modified by using 1 ml. of 10% (w/v) orthophosphoric acid, instead of 0·5 ml. of syrupy phosphoric acid, and using the apparatus of Markham (1942). It was found more convenient to use a direct-reading pH meter, rather than an indicator, for following the titration of the distillate. Volatile fatty acids were identified by paper chromatography with the method of Reid & Lederer (1952).

Glucose. The disappearance of glucose was followed with the anthrone method as modified by Trevelyan & Harrison (1952).

RESULTS

Effect of pH *on the rate of evolution of carbon dioxide from pyruvate.* When washed suspensions of *L. brevis* were incubated with pyruvate a gas, identified as CO_2 by its absorption in 20% (w/v) KOH, was evolved. With 0·1M-phosphate–citrate buffers the initial rate of CO_2 evolution was determined at several pH values in the range 3·5–6·5, with air and with N_2 as gas phases. The results shown in Fig. 1 reveal a pH optimum at 4·8 for both aerobic and anaerobic conditions. Subsequent experiments were therefore carried out at this pH.

Cofactor requirements. The effect of various likely cofactors on the rate of CO_2 evolution was tested in a series of experiments (Table 1). It is evident that both thiamine and Mn^{2+} ions were necessary

for maximum rate of CO_2 evolution when *L. brevis* was incubated with pyruvate. Replacement of Mn^{2+} by Mg^{2+} ions resulted in an 11 % lowering of the rate of CO_2 evolution. In subsequent experiments thiamine and Mn^{2+} ions (as $MnSO_4$) were added to all reaction mixtures.

Aerobic metabolism of pyruvate. When *L. brevis* was incubated with pyruvate there was no absorption of O_2 until an amount of CO_2 corresponding to 0·5 μmole of CO_2/μmole of pyruvate had been evolved. After this initial rapid reaction O_2 was absorbed and a further corresponding amount of CO_2 liberated, as can be seen from Fig. 2. The Warburg flasks at the conclusion of the experiment, and immediately after the primary rapid reaction, were examined for acetoin and α-acetolactic acid since, on the basis of similar earlier work by Juni (1952), these were considered to be likely intermediates. Neither compound was detected.

In order to attempt to establish the pathway of pyruvate breakdown in this micro-organism, Warburg flask contents were examined at suitable intervals for lactic acid, volatile fatty acids and residual pyruvate. From Fig. 2 it can be seen that all the pyruvate was utilized before the commencement of the O_2-absorbing reaction and that the lactate concentration reached a maximum at this point. The final flask contents, when acidified and steam-distilled, yielded amounts of volatile acid approximately equivalent to the initial concentration of pyruvate. The volatile acid was subsequently identified as acetic acid by paper chromatography.

The possibility that acetate was present in the form of acetyl phosphate in the final reaction mixture was visualized. Tests by the method of

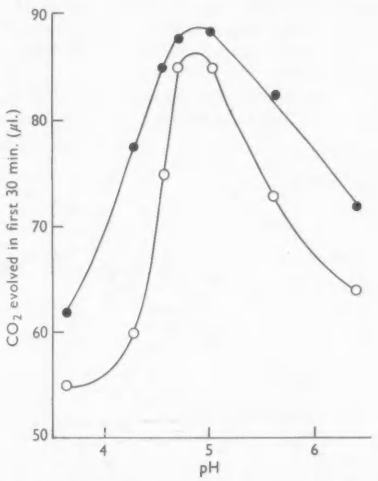

Fig. 1. Effect of pH on the rate of CO_2 evolution from pyruvate. Reaction mixture (3 ml.) contained 10 μmoles of pyruvate, thiamine, $MnSO_4$, phosphate–citrate buffer and 11 mg. of cells dry wt. Gas phase was air (○) or N_2 (●).

Table 1. *Effect of various cofactors on the rate of evolution of carbon dioxide from pyruvate*

Warburg flasks contained 10 μmoles of pyruvate, cofactors (0·1 ml. of each, 0·01 M) as stated below, 7 mg. of cells (dry wt.) and the volume was made up finally to 3 ml. with phosphate–citrate buffer, pH 4·8. Gas phase was nitrogen.

Cofactors added	Rate of CO_2 liberation (μl. in first 30 min.)
None	38
Thiamine	62
$MnSO_4$	63
$MgSO_4$	55
Thiamine + $MnSO_4$	74
Thiamine + $MnSO_4$ but no pyruvate	5

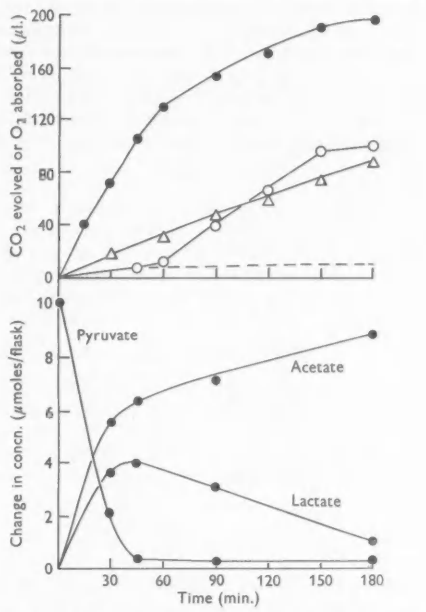

Fig. 2. Aerobic metabolism of pyruvate. Upper diagram: ○, △, O_2 uptake; ●, CO_2 evolved. Cells (10 mg. dry wt.) were incubated in 3 ml. of solution containing phosphate–citrate buffer, pH 4·8, thiamine, $MnSO_4$ and the following substrates: 10 μmoles of pyruvate (○); 5 μmoles of lactate (△). – – –, No substrate. The lower diagram records the changes in concentration of pyruvate, lactate and acetate throughout the experiment.

Lipmann & Tuttle (1945), however, gave negative results.

Added DL-lactate was slowly oxidized at a rate compatible with the slow O_2-absorbing reaction.

Anaerobic experiments. Experiments carried out in atmospheres of N_2 or N_2+CO_2 (95:5) with different amounts of pyruvate (see Fig. 3) gave values for CO_2 evolution of 0·8 molecule of $CO_2/2$ molecules of pyruvate. This value agrees approximately with that for the initial CO_2-evolving reaction (before the onset of O_2 absorption) of the aerobic experiments detailed above. The final flask contents were examined for acetoin, lactate and volatile acid. From an initial 10 μmoles of pyruvate, 4·1 μmoles of acetate and 4·0 μmoles of lactate, but no acetoin, were recovered.

Effect of added glucose. Rowatt (1951) and Nossal (1952) both found that for their systems the rate of CO_2 evolution from pyruvate was increased in the presence of glucose. Fig. 4 indicates that suspensions of *L. brevis* incubated with glucose as sole substrate showed an immediate uptake of O_2 with a corresponding evolution of CO_2. When pyruvate and glucose were both present O_2 was not absorbed until the rapid CO_2-liberating reaction had occurred; thereafter there was an uptake of O_2 and a corresponding further CO_2 evolution. This suggested that glucose oxidation was inhibited in the presence of pyruvate and this was confirmed by measuring the disappearance of glucose after suitable time intervals. From an initial 2 μmoles of glucose, in the presence of 10 μmoles of pyruvate (see Fig. 4), 1·8 μmoles of glucose was still present after 30 min., but no glucose remained at the conclusion of the experiment.

Effect of various inhibitors. With reaction mixtures containing pyruvate, thiamine and Mn^{2+} ions, the effects of the following inhibitors were studied: 0·01 M-fluoride, 0·01 M-azide, 0·01 M-arsenite, 0·01 M-arsenate and 0·001 M-fluoropyruvate. Fluoride, azide and arsenate did not inhibit pyruvate metabolism at these concentrations.

The effect of arsenite is shown in Table 2 and Fig. 5. This compound caused a slight lowering of the initial rate of CO_2 evolution but the O_2-absorbing reaction was markedly inhibited. Examination of the flask contents revealed an accumulation of acetoin and lactate in those flasks containing arsenite.

Fig. 5 also shows that fluoropyruvate caused an inhibition of the O_2-absorbing reaction similar to

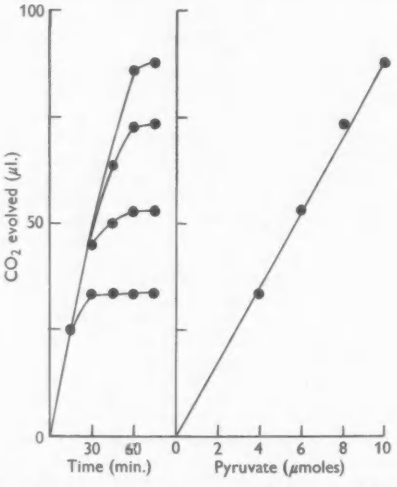

Fig. 3. Relationship between pyruvate concentration and CO_2 production. Cells (10 mg. dry wt.) were incubated in 3 ml. of solution containing phosphate–citrate buffer, pH 4·8, thiamine, $MnSO_4$ and different amounts of pyruvate, in an atmosphere of nitrogen.

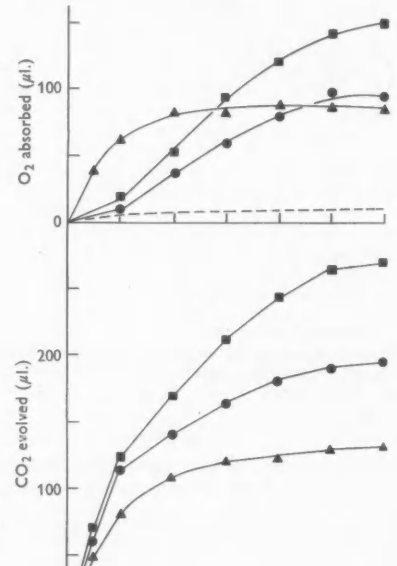

Fig. 4. Aerobic metabolism of pyruvate and glucose. Cells (12 mg. dry wt.) were incubated in 3 ml. of solution containing phosphate–citrate buffer, pH 4·8, thiamine, $MnSO_4$ and the following substrates: 10 μmoles of pyruvate (●); 10 μmoles of pyruvate + 2 μmoles of glucose (■); 2 μmoles of glucose (▲). – – –, No substrate.

that caused by arsenite but at one-tenth of the concentration. Fluoropyruvate poisoning likewise resulted in an accumulation of acetoin and lactate.

Substrate specificity. The substrate specificity of the keto acid-utilizing enzymes present in *L. brevis* was investigated by using 2-oxobutyrate or 2-oxoglutarate in place of pyruvate in Warburg experiments. It was found that 2-oxobutyrate was readily utilized but that 2-oxoglutarate was unaffected by suspensions of *L. brevis*. In one such experiment 10 μmoles of 2-oxobutyrate yielded 8 μmoles of volatile acid with the corresponding evolution of 8·4 μmoles of CO_2 and with an absorption of 4·4 μmoles of O_2. In this experiment the volatile acid was identified as propionic acid. By contrast with pyruvate, 2-oxobutyrate did not give rise to an acyloin compound in the presence of arsenite, as evidenced by a negative Voges–Proskauer test.

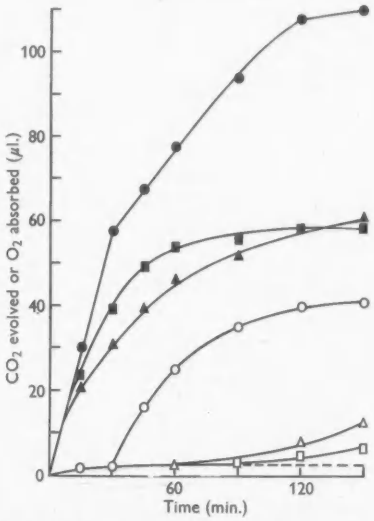

Fig. 5. Inhibition of pyruvate oxidation by arsenite and fluoropyruvate. Open symbols denote values for O_2 uptake, filled-in symbols values for CO_2 evolved. Cells (10 mg. dry wt.) were incubated in 3 ml. of solution containing phosphate–citrate buffer, pH 4·8, thiamine, $MnSO_4$, 6 μmoles of pyruvate (○, ●) or 6 μmoles of pyruvate + 0·01 M-arsenite (△, ▲) or 6 μmoles of pyruvate + 0·001 M-fluoropyruvate (□, ■). – – –, Endogenous respiration.

Confirmatory experiments with Lactobacillus brevis 6107. Experiments showed that *L. brevis* strain no. 6107, obtained from The National Institute for Research in Dairying, Reading, behaved in a similar manner to *L. brevis* strain 1·2 in Warburg experiments with pyruvate as substrate. Only when arsenite was added to the reaction mixture did acetoin accumulate. The reactions described in this paper are therefore not specific to *L. brevis* strain 1·2 but are probably characteristic of the *brevis* species.

DISCUSSION

The experiments reported here suggest that the metabolism of pyruvate in *L. brevis* occurs in two stages. On the basis of the results shown in Fig. 2 it is suggested that the initial reaction (i) corresponds to a dismutation

$$2\ \text{Pyruvate} \rightarrow \text{Lactate} + \text{acetate} + CO_2 \qquad \text{(i)}$$

$$\downarrow +O_2$$

$$\text{Acetate} + CO_2 \qquad \text{(ii)}$$

Under anaerobic conditions only reaction (i) normally occurs but in aerobic experiments reaction (i) was followed by a slower reaction (ii) involving oxidation of the lactate molecule to acetate. These reactions are in marked contrast with the reaction

$$2\ \text{Pyruvate} \rightarrow \text{Acetoin} + 2CO_2 \qquad \text{(iii)}$$

already established as the main pathway for pyruvate metabolism in the homofermentative lactobacilli (Rowatt, 1951; Nossal, 1952).

Hunt & Nossal (1954) showed that the stimulatory effect of glucose on the rate of evolution of

Table 2. *Final analyses of contents of Warburg flasks*

Flasks contained cells incubated in 3 ml. of solution containing 0·1 M-phosphate–citrate buffer, pH 4·8, thiamine, $MnSO_4$, pyruvate and inhibitor as stated. Reaction time was 180 min. This table records the results of two different experiments.

Initial pyruvate (μmoles)	Gas atmosphere	Inhibitor	CO_2 evolved (μmoles)	O_2 absorbed (μmoles)	Acetate (μmoles)	Lactate (μmoles)	Residual pyruvate (μmole)	Acetoin (μmole)
10	Air	None	8·8	3·9	8·8	0·8	0·1	None
10	N_2	None	4·0	0·0	4·1	4·0	0·2	None
6	Air	None	4·9	1·9	4·7	0·6	0·1	None
6	Air	0·01 M-Arsenite	2·7	0·05	2·1	2·3	0·2	0·65
6	Air	0·001 M-Fluoropyruvate	2·7	0·0	2·6	2·7	0·1	0·50

carbon dioxide from pyruvate by *L. arabinosus* was concomitant with its glycolysis to lactic acid. In the present system added glucose did not stimulate the rate of evolution of carbon dioxide and was not oxidized until reaction (i) was completed. This difference may be correlated with the finding of Buyze, Van de Hamer & De Haan (1958) that heterofermentative lactobacilli lack aldolase and operate the hexose monophosphate-shunt mechanism for glucose oxidation.

The action of arsenite in this system is unusual and difficult to explain since arsenite is usually considered as an inhibitor of oxidative decarboxylation. It is possible that both reactions (i) and (ii) involve oxidative decarboxylation but the latter is very much more sensitive to the poison than the former. The inhibitory action of fluoropyruvate may be due to a blockage of an enzyme system by a 'lethal synthesis' of a fluoro-substituted compound, or (a more acceptable hypothesis) to the greater sensitivity of the second reaction (ii) to the poison than the acetoin-forming system. Dolin & Gunsalus (1951), in the course of their studies with *Streptococcus faecalis*, reported the presence of two pathways for pyruvate metabolism in this organism. Acetate was found to be the main product of pyruvate breakdown by intact cells of *S. faecalis*, but an acetoin-synthesizing system, not apparent during whole-cell studies, was demonstrated in cell-free extracts. These workers also found that under anaerobic conditions *S. faecalis* formed acetate and lactate from pyruvate by the reaction:

$$2\ \text{Pyruvate} \rightarrow \text{Acetate} + \text{lactate} + CO_2$$

A latent acetoin-forming system appears to be present also in *L. brevis*, demonstrable only in the presence of suitable inhibitors of the alternative pathways.

When *L. brevis* was incubated with 2-oxobutyrate in the presence of arsenite, no acyloin compounds (propioin) were formed. Therefore it may be concluded that the acyloin-forming system is specific for pyruvate. A similar high degree of specificity for an acyloin-condensation system in *Aerobacter aerogenes* has been reported by Juni (1952).

The main conclusions reached therefore are that at least two pathways for pyruvate metabolism exist in *L. brevis*. The main route involves an anaerobic dismutation of 2 molecules of pyruvate to form lactate, acetate and carbon dioxide followed by oxidation of the lactate to acetate and carbon dioxide, a mechanism not exhibited by the homofermentative lactobacilli. These species synthesize acetoin from pyruvate, a reaction that is also available to *L. brevis* but is not of primary metabolic importance to this species.

SUMMARY

1. In contrast with the homofermentative lactobacilli, which are known to form acetoin as the main product of pyruvate metabolism, suspensions of *Lactobacillus brevis* metabolized pyruvate, to form ultimately acetate and carbon dioxide, in two distinct stages.

2. The first stage involved a dismutation reaction of pyruvate to form acetate, lactate and carbon dioxide. Under aerobic conditions the lactate was subsequently oxidized to acetate and carbon dioxide.

3. The rate of carbon dioxide evolution was maximum at pH 4·8. Thiamine and manganese (Mn^{2+} ions) stimulated the rate of carbon dioxide formation from pyruvate.

4. Oxidation of glucose, added to the above system, was suppressed until the dismutation reaction was completed.

5. When arsenite or fluoropyruvate was present in the system the second-stage oxygen-consuming reaction was suppressed and acetoin accumulated.

6. *Lactobacillus brevis* produced propionate from 2-oxobutyrate by a similar mechanism, but propioin accumulation could not be demonstrated in the presence of arsenite.

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